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PRINCIPAL INVESTIGATOR: George C. Prendergast, Ph.D.

CONTRACTING ORGANIZATION: Lankenau Institute for Medical Research
Wynnewood, PA 19096

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George C. Prendergast, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lankenau Institute for Medical Research
Wynnewood, PA 19096

E-Mail: Prendergastg@mlhs.org

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13. ABSTRACT (Maximum 200 Words)

This project seeks to determine the consequences of deleting Bin1, a gene encoding a Myc-interacting adapter protein with features of a tumor suppressor, for normal development or neoplastic transformation of the mouse prostate. In Year 2 of the project, we identified and resolved by use of an alternate strategy a pitfall in the use of the initial 'knockout' mouse strains obtained. Specifically, the Cre-mediated strategy for Bin1 knockout in mouse prostate was found to be inoperational in the strains used. As an alternate approach, we have generated mosaic mice that are heterozygous or nullizygous for Bin1 through the animal, including in the prostate. Mice that are highly mosaic for Bin1 knockout in the prostate are being monitored currently for effects on prostate development and tumorigenesis. Additionally, we crossed an activated Myc allele into the mosaic lineage to generate mice that have sustained Myc lesions and frequent Bin1 knockout in the prostate. These mice are being monitored currently for effects of Bin1 loss on conversion of Myc-induced prostatic intraepithelial neoplasia (PIN) to frank carcinoma.

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Introduction

In this project, we have proposed to test the central hypotheses that the Bin1 gene is required for normal development of the prostate and to restrain the initiation or progression of prostate cancer. The foundations of this central hypothesis were two-fold. First, Bin1 encodes an adapter protein that inhibits the transforming activity of the Myc oncoprotein, the activation of which drives a large fraction of prostate cancers (1-8). A large body of evidence supports the likelihood that Bin1 can function to promote differentiation, to facilitate stress signaling, and to restrain malignant development (9-18). Furthermore, several lines of evidence argue that in certain transformed cell settings Bin1 can facilitate a p53-independent mechanism cell suicide that can be triggered by Myc and other proapoptotic stimuli (14, 15). Loss of Bin1 may therefore relieve blocks to malignant development that involves a mechanism of differentiation and cell death. The second foundation to the central hypothesis tested in this project is that we have found Bin1 to be attenuated very frequently in human prostate cancers (12, 16) (McDonnell, T.J., Do, K.-A., Troncoso, P., Wang, X., Bueso-Ramos, C., Coombes, K., Brisbay, S., Lopez, R., Prendergast, G.C., and Logothetis, C. Application of data modeling strategies to prostate cancer tissue microarray: an analysis of selected cell cycle and cell death regulatory proteins. Manuscript submitted.) Thus, the restraint provided by Bin1 to the development of prostate malignancy may be relieved at some point during tumor development, possibly relieving the 'death penalty' which is associated with Myc activation in this setting (19).

While an unambiguous function for Bin1 has yet to be defined, when taken as a whole the literature suggests that this gene encodes a scaffold that acts to integrate signaling and trafficking processes in cells, perhaps crucial in cells under stress. With regard to cancer development, a key question is whether the common attenuation of Bin1 expression in cancers is derivative or causative with regard to tumor initiation or progression. This key question can be addressed using knockout mouse models, where a causative role can be established by demonstrating that gene ablation can promote one or both of these processes. In this project, a conditional knockout model based on

well-established cre-lox technology is being used. Briefly, we constructed a mouse strain in which Bin1 function can be ablated specifically in the prostate at the age of sexual maturation (~7 wks), by an allelic deletion that is generated by prostate-specific induction of Cre recombinase. Through this strategy, we can monitor the consequences of Bin1 loss at early times on androgen-driven development and homeostasis of the prostate (Aim 1). If Bin1 loss is sufficient to promote tumor formation, then at later times one would expect an elevation in the formation of preneoplastic or neoplastic lesions (Aim 1). If, alternately, Bin1 loss does not promote tumor initiation, but is instead sufficient to drive tumor progression, then one would expect an increase in the progression of pre-neoplastic or neoplastic lesions that are initiated by a separate event, in this case, by Myc activation (Aim 2). These are distinct aims, because it is readily conceivable that while Bin1 may have no role in development, homeostasis, or tumor initiation, it may be still have a critical role in limiting tumor progression (e.g. by stanching survival, motility, angiogenesis, or immune rejection after the tumor has developed). Indeed, preliminary studies suggest that Bin1 has a predominant role in limiting cancer at the level of progression, by acting as a negative modifier of survival but also of immune evasion of neoplastically transformed cells (20).

Body

Previous progress. Year 1 of this project was consumed mainly by performing breeding required to generate the desired experimental strains. In that phase of the work, as described in last year's progress report, we confirmed three important expectations of the system (briefly summarized here). First, we confirmed that the 'floxed' allele of Bin1 that had been generated could phenocopy the wild-type allele (before Cre-mediated deletion). Second, we confirmed was that the 'floxed' allele could successfully convert to the 'floxΔ' allele in vivo, in mice where the Cre gene was co-expressed. Lastly, we confirmed was that the floxΔ allele was in fact a functional knockout allele for Bin1. During Year 1 we also generated mosaic offspring in which different tissues displayed mixed KO/flox and KO/floxΔ genotypes. As discussed further below, these mosaic mice have turned out to be very important because they have

provided a way around an unexpected pitfall in the initial experimental model that emerged in Year 2.

Emergence of a pitfall and development of an alternate strategy in Year 2. Last year we achieved Task I of Objective I and Tasks I and II of Objective II, namely, to generate mice of the desired genotypes. However, in Year 2 we learned that the tet-regulated Cre transgene used for prostate-specific Bin1 knockout was in fact defective in operation. In the PB-cre transgenic mouse strain employed, Cre expression was directed specifically to prostate cells under control of the tet regulator doxocycline (added to drinking water at the desired time of induction). Unfortunately, we were unable to achieve the desired Bin1 knockout due to a lack of Cre induction in the prostates of the PB-cre;KO/+ and PB-cre;KO/flox strains and also the PB-cre;PB-myc;KO/+ and PB-cre;PB-myc;KO/flox strains that had been generated. Despite efforts to resolve this issue by several approaches (increased doxycycline adminstration, different routes of administration, using different litters of mice, using mice of different ages), we were not able to achieve Cre induction, as measured by RT-PCR, nor Bin1 knockout (as would be expected due to the lack of Cre induction). It remains unclear whether this problem derives from the mixed strain background of the strains that were generated, which differ from the original PB-cre strain, or from some other problem. In any case, this problem prevented us from accomplishing the remaining Tasks in Objectives I and II as originally proposed.

Construction of mosaic Bin1 knockout mice. The mosaic KO/flox strains that we created in Year 1 as a side project offered an alternate strategy to complete this proposal. Briefly, we generated KO/flox mosaic mice that are composed of a mixture of Bin1 null and Bin1 heterozygous cells throughout the animal. These animals were derived from crossing an EIIa-cre transgene onto the Bin1 KO/flox genotype. The EIIa promoter (derived from the EII gene of adenovirus) is known to be expressed only during E1-E4 of mouse development (21). Thus, in the EIIa-cre;KO/flox mice the transient activation of Cre resulted in Bin1 knockout (i.e. generation of a KO/floxΔ genotype) in some cells but not all cells of the resultant mouse. The generation of these

mice has been described in a manuscript that is not yet submitted but that is included for reference in the Appendix. Consistent with previous reports of the effects of EIIa-cre on 'floxed' gene recombination (21), we observed that different EIIa-cre;KO/flox mice have different levels of Bin1 gene knockout throughout various tissues, but that within each individual mouse the percent knockout across tissues is fairly uniform. Figure 1 presents the flox gene organization and the PCR primers used for genotyping the normal and floxed allele of Bin1. Figure 2 presents a comparison of the genotypes of mosaic and 'straight' knockout animals (left panel) and the tissue uniformity in the genotypes from highly or weakly mosaic individuals that are generated.

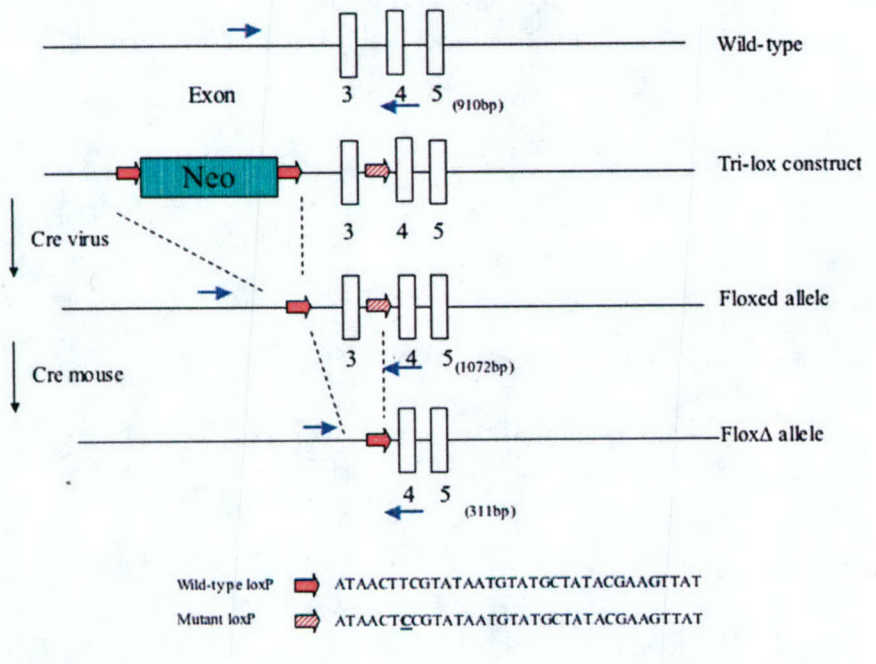

Figure 1. Bin1 flox gene construction and organization. Wild-type allele is shown at top. Blue arrows show the relative location of PCR primers used for genotyping, with associated sizes of the DNA fragment sizes produced in each PCR reaction). Mice generated from ES cells following Cre-mediated excision of the neo cassette in the "Tri-lox construct" have the floxed allele (flox) that retains full function. Further Cre-mediated reduction of the gene generates the floxΔ allele that is a functional knockout.

Here we note that mosaic models offer the inherent advantage of a 'built-in control', because cells that are positive and negative are found in the same individual animal. This is useful for analysis of a tumor suppressor gene such as Bin1, where gene loss is would be expected to lead to preferential tumorigenesis in knockout cells as compared to adjacent wild-type cells (i.e. if Bin1 acts as a suppressor, tumors will tend to develop from null cells, whereas if Bin1 does not act as a tumor suppressor, tumors will develop from either null or heterozygous cells similarly).

Figure 2. Genotype of Bin1 mosaic mice. Left panel, genomic DNA isolated from toe clip of two 3 wk EIIa-cre;Bin1 KO/floxΔ^{mosaic} animals was subjected to multiplex PCR using primers presented in Figure 1. One animal exhibits a mosaic genotype in the toe clip tissues whereas the second exhibits a near-complete knockout genotype. Right panel, PCR performed on tissues from two different EIIa-cre;Bin1 KO/floxΔ^{mosaic} animals illustrates that differences in overall degree of mosaicism are similar between tissues of the same individuals.

We have exploited these mosaic Bin1 knockout mice as an alternate strategy to address the central questions of this proposal, namely, whether Bin1 loss is sufficient on its own to drive the formation of premalignant and malignant prostate lesions, and/or whether Bin1 loss will cooperate with Myc to drive progression of premalignant lesions that are initated by Myc.

Progress in the alternate experimental strategy. Following this alternate strategy, in Year 2 we completed the generation of the new strains of mice that were desired (equivalent to Task I, Objective I and Tasks I and II, Objective II). Briefly, using an EIIa-cre-based strategy, we completed the generation of cohorts of mosaic mice that are EIIa-cre;Bin1 KO/floxΔ^{mosaic} or EIIa-cre;PB-myc;Bin1 KO/floxΔ^{mosaic} and have begun to monitor them for formation of premalignant lesions or malignant tumor formation. Recent advances in our laboratory argue that Bin1 loss is insufficient to initate cancer

Recent advances in our laboratory argue that Bin1 loss is insufficient to initate cancer but sufficient to promote tumor progression (20, 22) (preprints of these papers in press are provided in the Appendix). Therefore, we predict that Bin1 loss will not cause prostate cancer unless Myc is also activated in the prostate.

Key Research Accomplishments

1. Discovery of a pitfall in the use of a PB-cre transgene to elicit prostate-specific knockout of a floxed Bin1 allele in a mixed PB-cre;Bin1 KO/flox strain.
2. Generation of Ella-cre;Bin1 KO/flox and Ella-cre;PB-myc;Bin1 KO/flox mouse strains that are mosaic for Bin1 knockout.

Reportable Outcomes

None this period.

Conclusions

A pitfall that emerged in Year 2 of the project was circumvented by the use of mosaic Bin1 knockout mice that were generated as a side-project in Year 1. By this alternate strategy, new strains of mice were created that have the capacity to address the central questions of the proposal. Studies will proceed to assess the effects of Bin1 loss on prostate homeostasis and the incidence of preneoplastic or neoplastic lesions in the prostate.

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nature medicine

[AU: Do you mean Bin1 is a tumor suppressor? If not, please reword title. If so, please use tumor suppressor.]

Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy

Alexander J Muller[1], James B DuHadaway[1], P Scott Donover[1], Erika Sutanto-Ward[1] & George C Prendergast[1,2]

Immune escape is a crucial feature of cancer progression about which little is known. Elevation of indoleamine 2,3-dioxygenase (IDO) in tumor cells can facilitate immune escape; however, it is not known how IDO becomes elevated nor how IDO inhibitors might be used to treat cancer. Here we show that IDO is under genetic control of *Bin1*, which is attenuated in many human malignancies. Mouse knockout studies indicate that *Bin1* loss elevates the STAT1- and NF-κB-dependent expression of IDO, driving escape of oncogenically transformed cells from T cell–dependent antitumor immunity. In MMTV-*Neu* mice, an established breast cancer model, we show that small-molecule inhibitors of IDO cooperate with cytotoxic agents to elicit regression of established tumors refractory to single-agent therapy. Our findings suggest that *Bin1* loss promotes immune escape in cancer through IDO deregulation, and inhibiting IDO-based immunomodulation can leverage [AU: If you mean increase, plase state. Have omitted 'safely' as can't extrapolate to humans.] the efficacy of cancer chemotherapy.

Immune cells create a complex cytokine environment that promotes cancer cell survival, angiogenesis, invasion and metastasis[1]. To survive in this environment, however, cancer cells expressing recognizable tumor antigens must evolve strategies to thwart immune detection and destruction[2]. Immune escape is thus a hallmark of cancer progression, but its underlying molecular genetic basis remains poorly understood. The interplay between immune escape and other hallmarks of malignant conversion, such as invasion and metastasis, is similarly obscure. Aggressive and disseminated cancers can be eradicated by an appropriately activated immune system, arguing that overcoming immune escape might have broad therapeutic impact, but this expectation has yet to be realized. Small-molecule drugs are of particular interest because of their relative advantages compared to biological agents in terms of production, delivery and cost. Yet few small molecules for stimulating antitumor immunity have been described.

Studies of the BAR adapter–encoding gene *Bin1* (also known as *Amphiphysin2*) indicate that it functions in cancer suppression[3–9]. Certain Bin1 adapter isoforms associate with endocytotic complexes[10], but evidence from gene knockouts in several species suggest that *Bin1* is not essential for endocytosis[11–13] [AU: Sentence OK as edited?]. Instead, Bin1 adapters may be important for vesicle trafficking[14], consistent with evidence that BAR domains not only bind membranes but sense [AU: Please provide non-anthromorphic term.] membrane curvature[15]. BAR adapter proteins may integrate signaling and trafficking processes, in some cases perhaps involving sites of action in the nucleus[4,16] [AU: OK as edited?]. Nuclear localization of some Bin1 isoforms is important for cancer suppression; however, there is little information about the relevant effector pathways or about the precise pathophysiological consequences of attenuating the expression of nuclear isoforms, as occurs often in human malignancies[3,5–8].

IDO is emerging as an important immunoregulatory enzyme. It catalyzes the initial rate-limiting step in tryptophan catabolism, which leads to the biosynthesis of nicotinamide adenine dinucleotide [AU: Addition of 'adenine' correct?]. By depleting tryptophan from local microenvironments, IDO can block activation of T lymphocytes, which are particularly sensitive to loss of this essential amino acid[17,18]. Notably, IDO is needed to prevent T cell–mediated rejection of allogenic concepti[19]. IDO is overexpressed in many cancers, where it has been implicated in immune escape[20,21]. But its importance to cancer progression and therapy has yet to be gauged fully. Here we identify a mechanism for IDO elevation in cancer, and we show how pharmacological inhibitors of IDO can be used in combination with cytotoxic chemotherapeutic agents to elicit regression of established tumors.

RESULTS

Bin1 loss promotes immune escape by tumor cells

Based on evidence of interaction between Bin1 and c-Myc (encoded by *Myc*)[3,4,9,22], we investigated the effects of targeted deletion of the *Bin1* gene[12] on the malignant phenotype of primary mouse skin epithelial cells (keratinocytes) cotransformed by *Myc* plus an activated allele of *Hras1* (referred to below as MRKECs). We confirmed *Bin1* genotype and transgene expression by PCR analysis and western blot analysis

[1]Lankenau Institute for Medical Research, [AU: Please provide full street address.] Wynnewood, Pennsylvania 19096 USA. [2]Department of Pathology, Anatomy & Cell Biology, Jefferson Medical College, Thomas Jefferson University, [AU: Please provide full street address.] Philadelphia, Pennsylvania 19106 USA. Correspondence should be addressed to G.C.P. (prendergasgt@mlhs.org).

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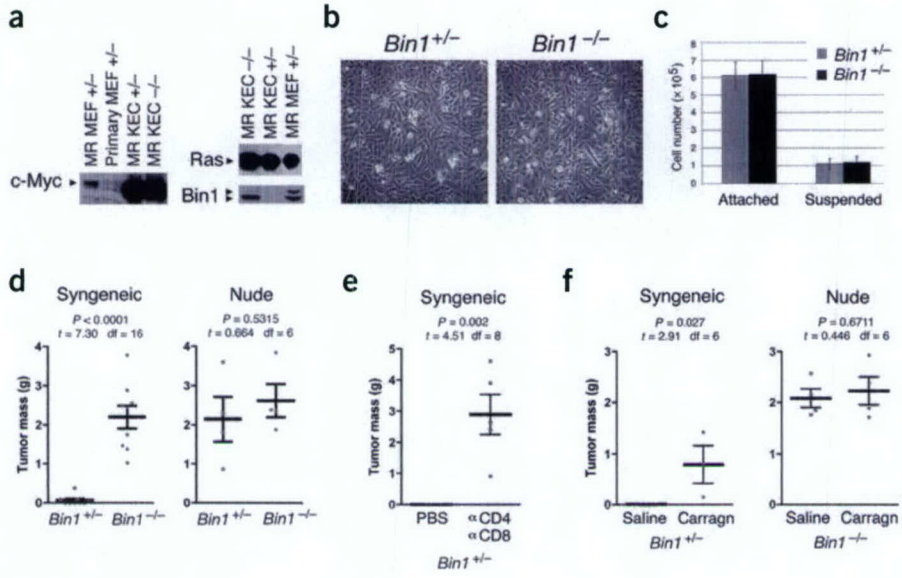


Figure 1 *Bin1* loss promotes tumor formation by facilitating immune escape. (**a**) Western blot analysis of MRKECs. (**b**) Cell morphology. (**c**) Cell proliferation. The experiment was performed twice. (**d**) Tumor formation in syngeneic versus nude mice. Tumor weight was determined 4 weeks after subcutaneous injection of MRKECs into syngeneic or nude mice. Each point on the graph represents a single tumor measurement with mean and standard error shown for each group (t = t ratio (the difference between sample means divided by the standard error of the difference between the means); df = degrees of freedom (n – 2)). **[AU: Should this be $n = 2$? What is n for all animal experiments?]** (**e**) Immune cell depletion phenocopies *Bin1* loss. $Bin1^{+/-}$ MRKECs were injected subcutaneously into mice depleted of both $CD4^+$ and $CD8^+$ cells and tumor formation was scored 3 weeks later. PBS, phosphate-buffered saline. (**f**) Carrageenan (Carragn) treatment partly phenocopies *Bin1* loss. $Bin1^{+/-}$ MRKECs were injected subcutaneously into control (saline-treated) and carrageenan-treated mice and tumor formation was scored 4 weeks later.

respectively (**Fig. 1a**). *Bin1* deletion did not alter the phenotype or *in vitro* proliferation of MRKECs under either anchorage-dependent or anchorage-independent conditions (**Fig. 1b,c**). Nevertheless, *Bin1* loss substantially enhanced the outgrowth of tumors formed by MRKECs in syngeneic animals (**Fig. 1c**). The significant difference in tumorigenicity between $Bin1^{-/-}$ and $Bin1^{+/-}$ cells ($P < 0.0001$) could not be explained by increased intrinsic cell proliferation, because $Bin1^{+/-}$ cells were no less aggressive at forming tumors than $Bin1^{-/-}$ cells in T cell–deficient, athymic nude mice (**Fig. 1d**) or in syngeneic mice depleted of both $CD4^+$ and $CD8^+$ T cells (**Fig. 1e**). Because tumor cells present antigens in part by cross-priming to [**AU: Should 'to' be 'of'?**] antigen-presenting cells (APCs), we investigated the effect of *Bin1* loss on tumors formed in syngeneic mice treated with carrageenan, a substance that depletes or inactivates phagocytic cells including macrophages, dendritic cells and neutrophils[23]. Carrageenan treatment enhanced tumor formation by $Bin1^{+/-}$ cells but not by $Bin1^{-/-}$ cells (**Fig. 1f**), consistent with the idea that cross-priming is involved in the antitumor immune response. In summary, we conclude that *Bin1* suppresses tumor formation through a cell-extrinsic, immune-based mechanism that appears to be dependent on both T cells and APCs.

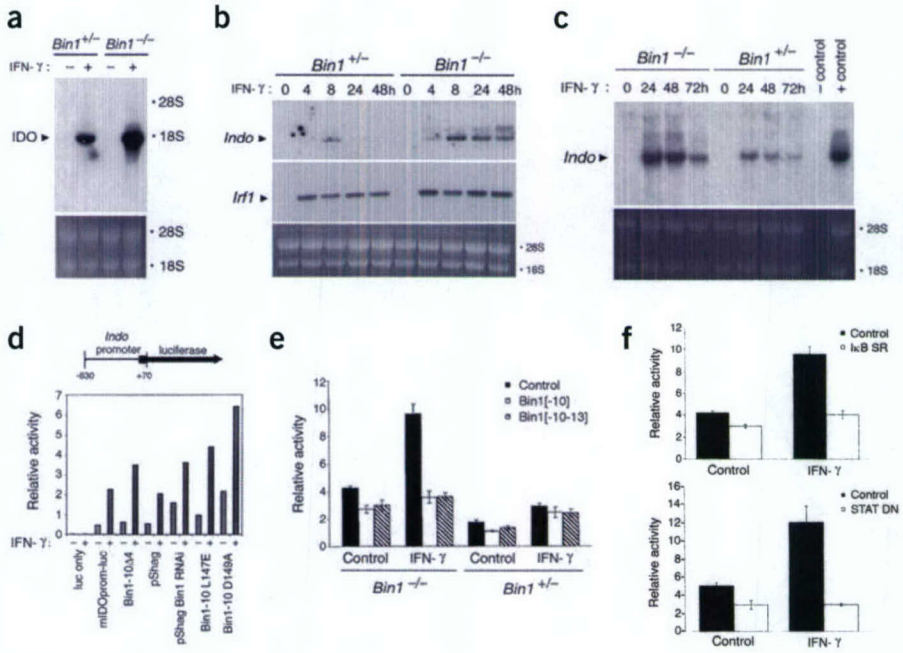


Figure 2 **[AU: Figure legend too long. Please move all relevant text to Results or Methods.]** Bin1 loss potentiates the NF-κB- and STAT-dependent expression of *Indo*. (**a**) *Bin1* loss elevates steady-state *Indo* levels after IFN-γ treatment. MRKECs were treated for 24 h with IFN-γ before RNA was isolated and analyzed by northern blotting with a mouse *Indo* cDNA probe. Ethidium bromide–stained gel is shown below as an RNA loading control. (**b**) *Bin1* loss leads to persistent induction of *Indo*. MRKECs were treated with IFN-γ for times indicated and processed for northern blot analysis as above. (**c**) *Bin1* loss accentuates induction of *Indo* in *Myc*-immortalized macrophages. Cells were treated with IFN-γ for times indicated and processed for northern blot analysis as before. (**d**) *Bin1* attenuation potentiates *Indo* transcription in human cells. HeLa cells were transfected with a mouse *IDO* promoter-luciferase reporter plus the indicated expression vectors. The day after transfection, cells were either left untreated or treated 16 h with IFN-γ and cell extracts were processed for normalized luciferase activity. Similar effects were produced by the dominant inhibitory mutants Bin1[-10] DN [22], Bin1[-10] L147E, and Bin1[-10] D149A. **[AU: If siRNA or shRNA, please indicate.]** (**e**) *Bin1* deletion potentiates *Indo* transcription in MRKECs. Cells were transfected with the *Indo* promoter reporter used above plus the indicated expression vectors, then treated and processed for normalized luciferase activity as before. (**f**) NF-κB and STAT1 are required for superinduction of *Indo* in *Bin1*-null cells. $Bin1^{-/-}$ MRKECs were cotransfected with *Indo* promoter-reporter used above plus vectors expressing either no insert, a super-repressor mutant of IκB that prevents NF-κB activation (top), or the dominant negative mutant STAT1 Y701F (bottom), then treated and processed for luciferase activity as before.

IDO is under genetic control of *Bin1*

Most cancers express tumor antigens that render them sufficiently antigenic to elicit an effective immune response [AU: "effective" immune response seems an overstatement. Please rephrase.]. Therefore, tumors must evolve mechanisms to evade or suppress anti-tumor immunity to progress successfully. Two recent studies have suggested that *Bin1* functions may modulate subcellular trafficking of the STAT and NF-κB transcription factors, which have important roles in modulating immunity[9,24]. In considering common genetic targets of STAT and NF-κB, we identified IDO (encoded by *Indo*) [AU: Our policy is to use approved gene symbols. IDO changed to Indo throughout. Please check for consistency.] as a candidate that might explain the effects of *Bin1* loss on immune escape. IDO is an extrahepatic oxidoreductase. In APCs, the expression of IDO is strongly elevated by interferon-γ (IFN-γ)[17,25]. Deletion of *Bin1* in MRKECs markedly increased IFN-γ-induced expression of IDO both quantitatively and temporally, such that the *Indo* message level was both higher and persisted longer than in *Bin1*-expressing cells (**Fig. 2a,b**). IDO was also superinduced by IFN-γ in $Bin1^{-/-}$ macrophages[12], showing a similar effect of *Bin1* deletion in a cell lineage in which IDO expression is physiologically relevant [AU: Wording is awkward.] (**Fig. 2c**). Transcription assays in human HeLa cells showed that attenuation of *Bin1* by either siRNA or dominant inhibitory strategies increased basal and IFN-γ-induced activity of the *Indo* promoter (**Fig. 2d**). Similarly, *Bin1* deletion elevated basal and IFN-γ-induced activity of the *Indo* promoter in MRKECs, and these effects were reversed by ectopic expression of *Bin1[-10]* or *Bin1[-10-13]* [AU: Please indicate significance of numbers in brackets.] cDNAs encoding the two Bin1 proteins that are ubiquitously expressed (**Fig. 2e**) [AU: Wording unclear.]. Activation of the *Indo* promoter requires the activity of STAT1 and NF-κB transcription factors[26], the regulation of which may be influenced in part by Bin1 functions[9,24]. We observed that the benefits of *Bin1* loss to IDO transcription were abolished by introduction of a 'super-repressor' mutant of IκB, which prevents NF-κB activation, as well as by a dominant negative STAT1 mutant, which prevents STAT1 activation (**Fig. 2f**). Taken together, these results indicate that *Indo* expression is under the genetic control of *Bin1* at the level of NF-κB- and STAT1-dependent transcription.

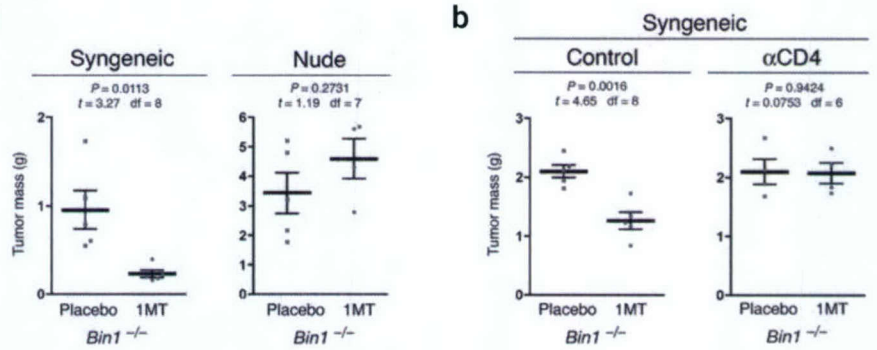
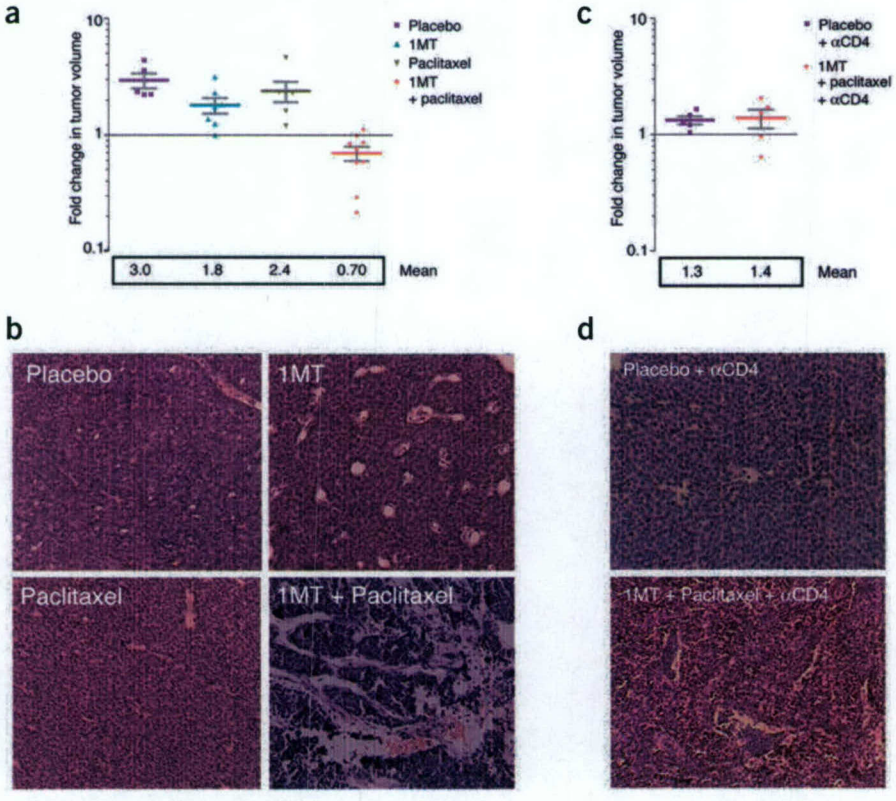


Figure 3 IDO inhibition counteracts the benefit of *Bin1* loss to tumor formation. (**a**) 1MT inhibits tumor formation by $Bin1^{-/-}$ cells. Mice implanted with either 1MT or placebo time-release pellets were injected subcutaneously with $Bin1^{-/-}$ MRKECs and tumor formation was scored 2 weeks later. (**b**) 1MT activity is abolished by T-cell depletion. Tumor formation was initiated and scored as before after subcutaneous injection of $Bin1^{-/-}$ MRKECs into mice treated with phosphate-buffered saline or CD4-specific monoclonal antibodies. **[AU: What is n for all animal experiments?]**



IDO mediates immune escape by tumor cells that lack *Bin1*

To determine the importance of IDO activity to immune escape caused by *Bin1* loss, we asked whether the IDO inhibitor 1-methyl-tryptophan (1MT) could specifically counteract the benefit of *Bin1* loss to MRKEC tumor growth in syngeneic mice. We confirmed that

Figure 4 IDO inhibition cooperates with paclitaxel to cause regression of autochthonous MMTV-*Neu* breast tumors. (**a**) Therapeutic response. Tumor-bearing MMTV-*Neu* mice were implanted with time-release pellets containing 1MT (20 mg/d) or placebo pellets. The next day, either paclitaxel (13.3 mg/kg [MTD]) or vehicle was delivered three times per week as an intravenous bolus dose. Tumor volumes were calculated 2 weeks after therapy was initiated. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group. (**b**) Tumor histology at endpoint. (**c**) Immune depletion abolishes the efficacy of the combination therapy. Tumor-bearing mice depleted of $CD4^+$ cells were treated with either combination therapy or vehicles and tumor volumes were calculated 2 weeks later. (**d**) Tumor histology at endpoint in immune-depleted mice. **[AU: Please provide n values for mouse expts. and magnification for b–d.]**

Table 1 IDO inhibition enhances the efficacy of certain commonly used cancer chemotherapeutic agents

Compound	Class	Mean ± s.e. (+1MT)	Mean ± s.e. (–1MT)	*P*	*n*	Dose (mg/kg)	Route	Schedule
Cisplatin	Alkylating agent	0.77 ± 0.18	1.7 ± 0.33	0.0419	7,8	1.0	i.v.	3×/week
Cyclophosphamide	Alkylating agent	0.81 ± 0.12	1.4 ± 0.18	0.0269	5,5	100	i.v.	3×/week
Doxorubicin	Antineoplastic antibiotic	0.79 ± 0.07	1.5 ± 0.25	0.0150	6,4	0.66	i.v.	3×/week
5-Fluorouracil	Antimetabolite	1.2 ± 0.20	1.1 ± 0.25	0.8926	8,7	50	i.v.	3×/week
Methotrexate	Antimetabolite	1.7 ± 0.28	1.7 ± 0.38	0.9047	3,3	1.0	i.v.	3×/week
Paclitaxel	Mitotic inhibitor (taxane)	0.68 ± 0.11	2.4 ± 0.43	0.0010	8,7	13.3	i.v.	3×/week
Vinblastine	Mitotic inhibitor (vinca alkaloid)	1.3 ± 0.19	1.2 ± 0.18	0.7368	10,8	1.0	i.v.	3×/week
FTI	Signal transduction inhibitor	0.67 ± 0.11	1.0 ± 0.16	0.0979	8,8	40	i.p.	qdx11
Rapamycin	Signal transduction inhibitor	0.97 ± 0.07	0.99 ± 0.25	0.9417	4,4	1.5	i.v.	qdx11
Tetrathiomolybdate	Antiangiogenic (iron chelator)	1.9 ± 0.52	2.0 ± 0.42	0.7996	3,4	40	p.o.	qdx11
Vehicle		1.7 ± 0.17	3.0 ± 0.44	0.0061	12,5			

Tumor-bearing MMTV-*Neu* mice were treated with either 1MT (+1MT) or placebo (–1MT) in combination with the cytotoxic drugs and molecular therapeutic agents indicated (at the doses indicated). We scored tumor volumes just before and 2 weeks after initiation of therapy. Fold changes in tumor volumes were determined and the means are presented for each group. i.v., intravenous; i.p., intraperitoneal; p.o., per os (orally) **[AU: OK as edited?]**; qdx11, **[AU: Please define qdx11.] [AU: P is between which groups? Please also define FTI and ensure that structure is available, otherwise must be removed.]**

the delivery method used (subcutaneous time-release pellets) could elicit maternal immune rejection of allogeneic but not syngeneic concepti (**Supplementary Fig. 1** online), indicating sufficient systemic exposure of 1MT to achieve biological activity. In tumor-bearing animals, 1MT inhibited the growth of *Bin1*$^{-/-}$ tumors in syngeneic hosts but not in nude hosts (**Fig. 3a**). Moreover, immune depletion of CD4$^+$ T cells from syngeneic animals abolished the ability of 1MT to suppress *Bin1*$^{-/-}$ tumor growth (**Fig. 3b**). 1MT does not seem to be directly cytotoxic or growth inhibitory, as it did not affect tumor growth in nude or immune-depleted syngeneic mice, nor were cytotoxicity or growth inhibition observed when MRKECs were treated with 1MT *in vitro* (data not shown). We conclude that IDO elevation is a critical mediator of immune escape caused by *Bin1* loss.

IDO inhibitors potentiate cancer chemotherapy

We next investigated whether IDO is critical for tumor survival in MMTV-*Neu* mice, a well-accepted transgenic mouse model of breast cancer, which, like human malignant breast cancers, shows attenuation of Bin1 expression (**Supplementary Fig. 2** online). MMTV-*Neu* mice bearing autochthonous tumors were randomly enrolled into control and treatment groups when tumors reached a diameter of 5–10 mm. 1MT was administered as in the MRKEC tumor graft setting described above. In some trials, we combined 1MT with paclitaxel, a chemotherapeutic agent used for breast cancer treatment, based on reports that taxanes promote T-cell infiltration of tumors[27]. By itself, 1MT retarded but did not arrest outgrowth of autochthonous tumors (**Fig. 4a**). Combinations with either IFN-γ or interleukin (IL)-12 did not accentuate the effect of 1MT (data not shown), arguing that IDO inhibition could not compromise the survival of established tumor cells, even when combined with immune stimulatory cytokines.

In marked contrast, combining 1MT with various cytotoxic agents led to tumor regressions under conditions where single agents were ineffectual. Combining 1MT with paclitaxel resulted, on average, in a 30% decrease in tumor volume within 2 weeks of initiating therapy, whereas paclitaxel delivered at near the maximum-tolerated dose (MTD) only slightly retarded tumor growth (**Fig. 4a**). Histopathological analysis of tumor sections from 1MT + paclitaxel–treated mice offered evidence of increased tumor-cell death (**Fig. 4b**). Immune depletion of CD4$^+$ T cells limited tumor growth relative to control tumors during the experiment but, as expected, it abolished the ability of 1MT + paclitaxel treatment to elicit tumor regression (**Fig. 4c**). This effect correlated with a reduction in tumor-cell death as evidenced by histological analysis of tumor sections (**Fig. 4d**).

It is unlikely that a drug-drug interaction simply caused an increase in the effective dose of paclitaxel, which was administered at near the MTD[28] **[AU: Please spell out.]**, because we did not observe characteristic neuropathies that would be expected to occur if 1MT had increased the effective dose (data not shown). Furthermore, this interpretation does not explain the effectiveness of combining 1MT with other drugs cleared by different mechanisms. We ruled out the trivial possibility that high doses of a tryptophan-like compound were sufficient by showing that DL-tryptophan **[AU: Is DL correct?]** was ineffective when substituted for 1MT in the regimen (data not shown). Lastly, we further confirmed the requirement for T cell–dependent immunity by showing in a tumor graft model, using an MMTV-*Neu*–derived tumor cell line, that 1MT showed effects only when the tumors were established in immunocompetent syngenic mice and was ineffectual in T cell–deficient athymic nude mice (**Supplementary Fig. 3** online).

We next evaluated the effects of combining 1MT with other cytotoxic drugs with diverse mechanisms of action that are used to treat breast cancer (**Table 1**). Among the agents tested were the DNA alkylating drugs cisplatin and cyclophosphamide, the topoisomerase inhibitor doxorubicin (adriamycin), the antimetabolites 5-fluorouracil and methotrexate, and the antimitotic agent vinblastine. We also tested several molecular targeted agents, including a farnesyl transferase inhibitor (FTI; L-744,832), the mTOR pathway inhibitor rapamycin,

and the angiogenesis inhibitor tetrathiomolybdate. 1MT cooperated with cisplatin, cyclophosphamide and doxorubicin to elicit mean tumor regressions that differed significantly ($P < 0.05$) from the impact of single-agent therapy (**Table 1**). Mean tumor regression was also produced in combination with FTI; however, as a result of the growth suppression produced by FTI alone, the differential with the combination treatment did not meet the threshold for assigning significance ($P > 0.05$; **Table 1**). We conclude that IDO inhibition cooperates with diverse chemotherapeutic agents to effectively promote regression of established breast tumors that are refractory to chemotherapy. [**AU: Please note that if the structure of FTI is not publicly available, data in table and comments in text must be removed.**]

A new inhibitor of IDO with antitumor activity

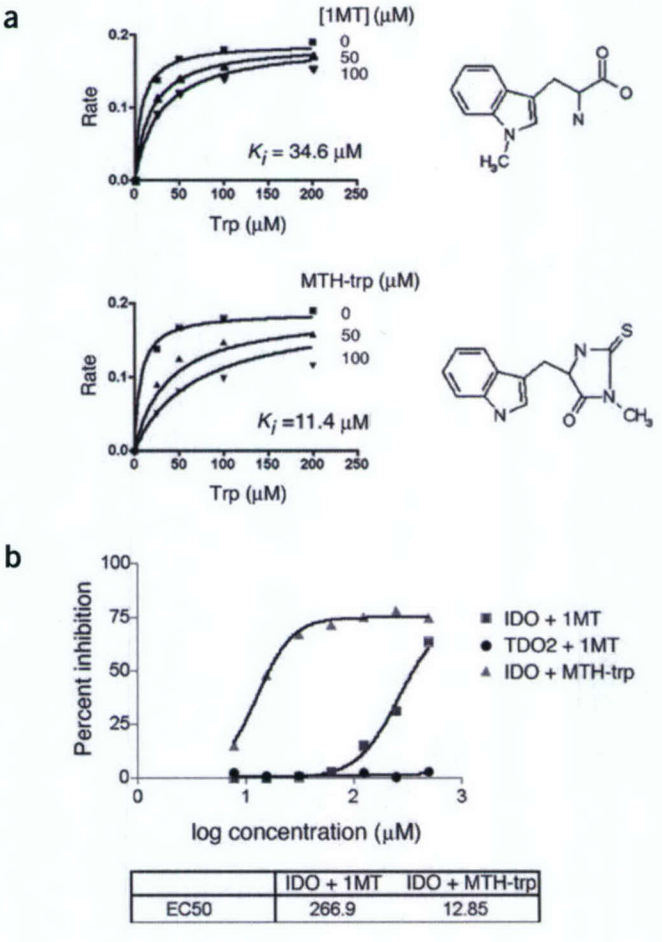
The observations described above are consistent with the presumptive specificity of 1MT for IDO; however, we wished to address off-target concerns by examining the ability of a structurally distinct inhibitor of IDO to elicit tumor regression in combination with paclitaxel. Toward this end, we screened for bioactive inhibitors among commercially available indoleamine-containing compounds, using a purified recombinant human IDO enzyme for *in vitro* assays and a human *INDO* cDNA for expression in cell-based assays. Both assays used a colorimetric method to quantify the production of kyneurinine, the product of reaction catalyzed by IDO[29]. Several compounds were identified that showed inhibition constants against recombinant human IDO that were within about two- to threefold of 1MT ($K_i = 34.2$ μM). One active compound was methyl-thiohydantoin-tryptophan (MTH-trp), which biochemical analyses showed to be a competitive inhibitor with $K_i = 11.6$ μM (**Fig. 5a**). Cell-based screens were performed after transient expression of human *INDO* cDNA in COS-1 monkey cells. As a counterscreen for selectivity, the inhibitory activity of the compounds against the structurally distinct liver enzyme TDO2 was also determined. MTH-trp was ~20-fold more potent than 1MT in the cell-based assay (**Fig. 5b**). Two other thiohydantoin derivatives of tryptophan with lower potency than MTH-trp were also identified (data not shown), confirming the IDO-inhibitory nature of this structural class and suggesting that the thiohydantoin sidechain is probably a mimetic of the amino acid backbone in tryptophan (**Fig. 5a**). Pilot pharmacology experiments have indicated that MTH-trp is more soluble in aqueous solution than 1MT but is also more rapidly cleared from serum; both compounds were found to be orally bioavailable (**Supplementary Fig. 4** online).

Using the same formulation, route of delivery and dose used previously to administer 1MT, we observed that combining MTH-trp with paclitaxel produced regression of tumors as well or better than 1MT (**Fig. 6a**). At the same 2-week endpoint, autochthonous tumors subjected to the combination therapy regressed, on average, 45% relative to the starting tumor volume. One mouse in the trial showed complete tumor regression. Histological examination of tumor sections confirmed evidence of tumor-cell death elicited by the combination therapy, as expected (**Fig. 6b**). Like 1MT, MTH-trp administered by itself retarded tumor outgrowth but did not promote regression. In addition, MTH-trp produced no evidence of gross toxicity in the mice during treatment or at necropsy. Compound titration showed that combinatorial efficacy was fully retained when the MTH-trp dose was reduced twofold and partly reduced when the dose was reduced fourfold (data not shown) [**AU: Fold reductions in this sentence unclear. Please restate using percentage or fraction.**]. The ability of MTH-trp to effectively combine with paclitaxel in a similar manner as 1MT strengthens the interpretation that the basis for this cooperativity is through IDO inhibition.

DISCUSSION

Immune escape is a central hallmark of cancer. Compared to other recognized hallmarks of cancer—immortalization, suppressor loss, sustained growth, apoptotic resistance, angiogenesis, invasion and metastasis[30]—much less is known about the genetics of immune escape. Here we address this gap in knowledge by defining a genetic mechanism that restricts the ability of cancer cells to escape T cell–dependent antitumor immunity. Using a mouse knockout model, we have shown that *Bin1* can restrain immune escape of oncogenically transformed cells by restricting expression of IDO, an immunoregulatory enzyme that is widely elevated in human cancer[21]. *Bin1* is likewise widely attenuated or mis-spliced in cancers of the breast, prostate, colon, brain and other organs[3,5–8,31,32] (K. Xie, L. Wang, J.B.D. and G.C.P., unpublished data). The finding that IDO is under negative genetic control by *Bin1* offers mechanistic insight into how immune escape may be enabled during cancer progression.

Translational studies prompted by our findings have led us to identify a new strategy for cancer treatment that combines IDO inhibitor–based immunomodulation with cytotoxic chemotherapy. Although gene abla-



	IDO + 1MT	IDO + MTH-trp
EC50	266.9	12.85

Figure 5 MTH-trp is a potent bioactive inhibitor of IDO. (**a**) *In vitro* enzyme assay. Global nonlinear regression analysis of enzyme kinetic data obtained for human IDO in response to 1MT and MTH-trp. Computed K_i values are shown for each compound. (**b**) Cell-based assay. Results of dose-escalation studies over two logs are shown for 1MT against both IDO and TDO2 and for MTH against IDO. EC_{50} values determined by nonlinear regression are shown.

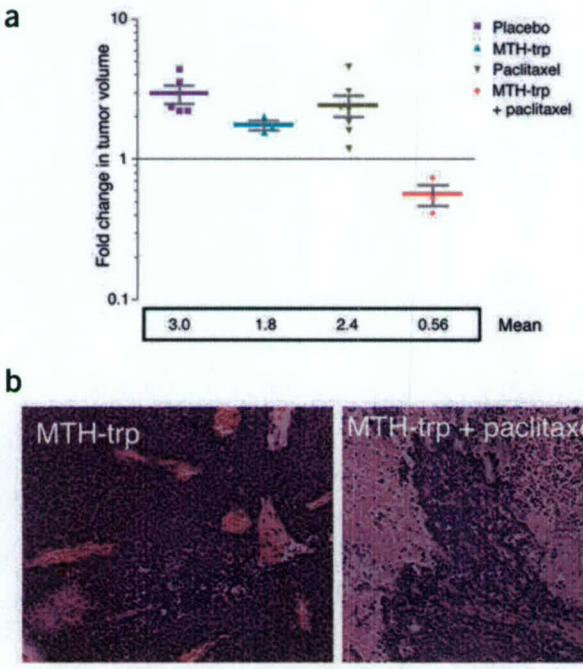


Figure 6 MTH-trp enhances paclitaxel efficacy. Tumor-bearing mice were implanted with time-release pellets containing MTH-trp (20 mg/d) pellets. Paclitaxel was administered and tumor responses were scored as described in **Fig. 4a**. Results from placebo control and paclitaxel-only treatment groups from **Figure 4** are provided for comparison. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group. The MTH-trp + paclitaxel group includes one complete tumor regression that cannot be plotted on the log scale. **[AU: Please specify info for panels a and b.] [AU: Please include n values and magnification.]**

tion studies are needed to fully validate IDO as a therapeutic target, the chemical genetics strategy used here offers an initial line of support. Combination drug treatment for cancer is the standard of care, but few studies have explored combinations of immunomodulating agents with chemotherapy. Our findings argue that immunotherapy and chemotherapy can be combined to more effectively destroy cancer cells, consistent with two other preclinical studies that focused on different immunotherapeutic principles[33,34].

Bin1 restrains IDO at the level of IFN-γ-regulated transcription by limiting the induction of IDO message by STAT1 and NF-κB, two key regulators of immunity and cancer. Precisely how *Bin1* influences the STAT1- and NF-κB-dependent transcription of *Indo* remains to be determined. Because nuclear localization is important for the suppressor activity of Bin1 isoforms [**AU: Provide reference?**], nuclear actions may be relevant. Because of a lack of mechanistic understanding, most studies of Bin1 adapter proteins have ignored evidence of nuclear localization of the ubiquitous isoforms[3,32,35,36], despite an established precedent for nuclear localization of other 'endocytotic-like' proteins (*e.g.*, epsin and CtBP, also known as BARs[37–39] **[AU: OK as edited? Please write out BAR.]**). Nuclear localization is also intriguing given intrinsic transcriptional repression activity associated with the Bin1 BAR domain[4] (M. Huang, P.S.D. and G.C.P., unpublished data). Indeed, a recent study of the BAR adapter protein APPL reinforces this concept by showing not only its nuclear trafficking function but also its association with the chromatin remodeling complex NuRD/Mi-2 that represses transcription[16]. In summary, effects on trafficking and/or transcriptional repression by *Bin1* may be relevant to its regulation of IDO.

This study extends the evidence that *Bin1* limits cancer pathophysiology and *Myc* oncogenicity[3–9,22,40,41]. Earlier work on cell-intrinsic suppressor roles of *Bin1* are expanded here by the identification of a cell-extrinsic suppressor role that involves restraining a protoleragenic mechanism that tumor cells can exploit to escape antitumor immunity. These cell-intrinsic and extrinsic suppressor roles might be intertwined given the complex involvement of the immune stromal environment in cancer pathophysiology. *Myc* overexpression is associated with major histocompatibility complex (MHC) class I downregulation in neuroblastomas and melanomas[42,43], two cancers in which *Bin1* is often attenuated by mis-splicing[5,8]. Thus, MHC class I downregulation may cooperate with Bin1-IDO dysregulation to facilitate immune escape by cells that overexpress *Myc*. The presence of IFN-γ in the tumor microenvironment may provide a selective pressure to explain the attenuation of *Bin1* and the upregulation of IDO during cancer progression.

This is the first study to link IDO to a cancer suppression pathway. Elevated tryptophan catabolism in cancer patients has been recognized for decades[44,45], a phenomenon that can be explained by IDO overexpression in tumors[21]. IDO catalyzes the initial step in tryptophan catabolism that leads to biosynthesis of nicotinamide adenine dinucleotide. But IDO does not catabolize dietary tryptophan, and because mammals salvage rather than synthesize nicotinamide adenine dinucleotide, the biological role of the enzyme was obscure until it was shown **[AU: Our style precludes mention of specific author names.]** that localized tryptophan catabolism forms the basis for a unique mechanism of establishing peripheral tolerance[19] **[AU: Sentence very long. Please break into two.]**. Because IDO inhibition is not inherently cytotoxic, identification of IDO inhibitors would elude traditional cytotoxic drug screens that are based on tumor-cell survival in tissue culture or in xenograft mouse model systems. In immunocompetent mice, we found that IDO inhibitors potentiated the efficacy of certain cytotoxic drugs without increasing their side effects. The mechanistic basis for the cooperation is not yet clear. Cooperating cytotoxic agents may induce certain types of cell death (*e.g.*, apoptotic versus nonapoptotic death) that elevate presentation of tumor antigens. Alternately, cooperating cytotoxic agents may preferentially compromise the survival of regulatory T cells relative to effector cells, contributing to a weakening of immune tolerance and stimulation of antitumor immunity[27,33,34]. In future work, it will be important to distinguish these possibilities, for example, by determining whether combinatorial efficacy is retained against drug-resistant tumor cells and whether the cytotoxic drugs that are effective in combination with IDO inhibition have similar effects on the immune system, despite their diverse cytotoxic mechanisms.

Our findings are consistent with the interpretation that IDO activity in tumor cells is the relevant target for inhibition; however, they are not incompatible with the possible involvement of stromal APCs, in which IDO can also be highly expressed (*e.g.*, as in the draining lymph nodes of breast tumors[46]). Thus, it is possible that IDO inhibitors may act in part by blocking an immune-tolerizing activity of APCs that are located at distal sites. From a therapeutic standpoint, the possibility that IDO may be a stromal target increases its appeal because of the reduced likelihood of selecting for drug resistance (relative to genetically plastic tumor cells), and because of the increased range of tumors that might be treated even in the absence of IDO overexpression. In closing, this study proposes IDO as an attractive and tractable target for the development of small-molecule immunomodulatory drugs to safely leverage the efficacy of standard chemotherapeutic agents.

METHODS

Drugs and chemical compounds. We purchased drugs as clinical formulations

or formulated them as described in **Supplementary Methods** online. For *in vitro* studies, 1MT (Sigma) and MTH-trp (Sigma) were formulated in dimethyl sulfoxide (DMSO), including 0.1 N HCl for the less soluble compound 1MT. For *in vivo* studies, 1MT and MTH-trp were formulated in 2-week time-release pellets (Innovative Research).

Tissue culture. We obtained and cultured primary skin keratinocytes from E18.5 days post-coitus **[AU: OK for dpc?]** mouse embryos on a mixed 129sv/BL6 background[12], essentially as described[47]. Transformed cell populations, referred to as MRKECs **[AU: OK as edited?]**, were generated by infection with ecotropic helper-free *Myc* and *Hras1* recombinant retroviruses and analyzed *in vitro* as detailed in **Supplementary Methods** online.

Northern and western blot analyses. We used standard methods as described in **Supplementary Methods** online. We generated a mouse monoclonal antibody recognizing the mouse and human IDO proteins (clone 10.1; UBI) essentially as described[48], using a bacterially expressed peptide encoding amino acids 78–184 of the human IDO protein.

Transcription assays. Cells seeded overnight in 12-well dishes were transfected with 200 ng mIDOprom900-luc, a luciferase reporter plasmid containing 900 base pairs of the mouse *Indo* promoter and 70 nucleotides of noncoding sequences in exon 1, 100 ng CMV-β-galactosidase (to normalize transfection efficiencies) and 700 ng of CMV-Bin1 plasmids as noted. Total DNA in each transfection was made up to 1,000 ng with the analogous CMV empty vector (pcDNA3-neo; Invitrogen). Detailed protocols for transfection and normalized reporter analysis are provided in **Supplementary Methods** online. **[AU: Please ensure that sequences for siRNA or shRNA are provided.]**

Tumor formation and drug response assays. For tumor formation by MRKECs, we injected 1×10^6 cells subcutaneously into syngeneic F1 offspring from 129S1/SvImJ and C57BL/6J breeders (Jackson Laboratories) and into immunocompromised CD-1 Nude (Crl:CD-1-nuBR) mice (Charles River Laboratories). Four weeks after cell injection, mice were killed **[AU: Our style precludes use of euphemisms for this.]** and tumor mass was determined. We generated autochthonous mammary gland tumors in multiparous female MMTV-*Neu* mice harboring the normal rat *HER2/Neu/ErbB2* gene (Jackson Laboratories). The incidence of detectable tumors in this model is ~80% at 7 months of age and increases to nearly 95% at 8 months. To monitor drug responses, we randomly enrolled tumor-bearing mice into control and experimental treatment groups when tumors reached 0.5–1.0 cm in diameter **[AU: OK as edited?]**. Based on the release rate computed by the vendor, the total dose delivered by subcutaneous time-release pellets was at least 20 mg/d, confirmed in pilot tests for a period of up to 5 d by pharmacokinetic analysis of blood serum. Control mice received placebo pellets only. Two days after pellet implantation, we delivered all chemotherapeutic agents except FTI and tetrathiomolybdate by bolus intravenous injection into the tail vein on a schedule of three times per week for a period of 2 weeks. FTI and tetrathiomolybdate were delivered daily by intraperitoneal injection on the same schedule. The doses used for each cytotoxic agent were at or near the MTD reported in the literature. At the 2-week endpoint, we determined tumor volume and wet weight. Tumors were both frozen and fixed and subsequently processed, sectioned and analyzed by standard methods as described in **Supplementary Methods** online. All methods involving mouse use were approved by the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research.

Immune cell depletions. Immunocompetent mice used in this study were subjected to cell depletion using standard methods[23,49]. Briefly, for T-cell depletion, we isolated tissue culture supernatants from the rat hybridomas GK1.5 and 2.43 and used them as a source of CD4-specific and CD8-specific monoclonal antibodies. Mice were injected intraperitoneally with 0.5 mg antibody for 3 d consecutively. We monitored splenic T-cell numbers 3 d later and at the experimental endpoint by flow cytometry using FITC-conjugated antibodies. Cell depletion was maintained during both tumor formation and tumor therapy experiments by intraperitoneal injection of 0.5 mg antibodies every 3 d. This strategy depleted >95% of the targeted T-cell subset in all treated mice compared to control mice that were injected with phosphate-buffered saline **[AU: OK for PBS?]**. For depletion of macrophages and APCs, we injected mice intraperitoneally with 2 mg carrageenan at 6, 3 and 1 d before subcutaneous injection of MRKECs, after which mice were injected one time per week up to the experimental endpoint.

IDO enzyme assays and inhibitor screens. We purified recombinant human his_6-IDO from *E. coli* strain BL21DE3pLys and used it in enzymatic reactions essentially as described[29]. The biochemical and cell-based inhibitor screening assays that were used to identify new IDO inhibitors were performed in a 96-well plate format, as described in detail in **Supplementary Methods** online.

Accession numbers. The GenBank accession numbers for the human IDO protein and the *Mus musculus* strain C57BL/6J chromosome 8 genomic contig, sequences 3011229–3010328 are NP 002155 and NT_039456, respectively.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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Research Paper

APPENDIX 2

Targeted Deletion of the Suppressor Gene Bin1/Amphiphysin2 Accentuates the Neoplastic Character of Transformed Mouse Fibroblasts

Alexander J. Muller[1]
James B. DuHadaway[1]
P. Scott Donover[1]
Erika Sutanto-Ward[1]
George C. Prendergast[1,2,*]

[1]Lankenau Institute for Medical Research; Wynnewood, Pennsylvania USA

[2]Department of Pathology; Anatomy and Cell Biology; Jefferson Medical College; Thomas Jefferson University; Philadelphia, Pennsylvania USA

*Correspondence to: George C. Prendergast; Lankenau Institute for Medical Research; Cancer Cell Signaling Laboratory; 100 Lancaster Avenue; Wynnewood, Pennsylvania 19096 USA; Tel.: 610.645.8475; Fax 610.645.8091; Email: prendergastg@mlhs.org

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KEY WORDS

Myc, BAR proteins, signal transduction, endocytosis

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NOTE

Supplementary material accompanies this manuscript. Supplementary Figure 1S and can be found at: http://www.landesbioscience.com/journals/cbt/muller3-12-sup.pdf.

ABSTRACT

The *Bin1/Amphiphysin2* gene encodes several alternately spliced BAR adapter proteins that have been implicated in membrane-associated and nuclear processes. *Bin1* expression is often attenuated during tumor progression and Bin1 splice isoforms that localize to the nucleus display tumor suppressor properties. While these properties may reflect the ability of these isoforms to interact with and suppress the cell transforming activity of c-Myc, the effects of *Bin1* deletion on the oncogenicity of *c-myc* or other transforming genes has not been gauged directly. Here we report that targeted deletion of *Bin1* enhances the neoplastic character of primary murine embryo fibroblasts (MEFs) cotransformed by *c-myc* and mutant *gras*g. Specifically, *Bin1* loss accentuated the spindle morphology of transformed cells, increased anchorage-independent proliferation, and promoted tumor formation in syngeneic hosts. These effects were specific as they were not recapitulated in cells transformed by viral oncoproteins and mutant *ras*. Although some Bin1 splice isoforms associate with endocytotic complexes the effects of *Bin1* loss were not correlated with a generalized defect in receptor-mediated endocytosis. However, *Bin1* loss increased sensitivity to paclitaxel, a drug that can affect endocytotic trafficking by disrupting microtubule dynamics. In E1A+*ras*-transformed MEFs, *Bin1* loss reduced the susceptibility to apoptosis triggered by tumor necrosis factor-α, an effect that was associated with precocious nuclear trafficking of NF-κB. These findings offer a novel line of support for the hypothesized role of *Bin1* in limiting malignant growth, possibly as a negative modifier or anti-progression gene.

INTRODUCTION

Cancer development requires the activation of oncogenes, the inactivation of tumor suppressor genes, and the modulation of gateway process that are controlled by modifier genes. During cancer progression, a selection against suppressor and negative modifier genes occurs due to the restraint they enforce to oncogene-driven proliferation and other processes. Myc is a major oncogenic driver in human cancer. At early times during cancer development, its deregulation supports cell division, whereas at later times its frequent overexpression drives malignant progression. Secondary events that abolish suppressor and negative modifier functions facilitate Myc oncogenicity and allow cells to become refractory to the intrinsic apoptotic penalties that are associated with inappropriate activation of Myc.[1] The nature of these suppressors and negative modifier functions is of interest, not only to understand how Myc drives cancer development but also to identify possible strategies to restore the penalties associated with Myc activation for therapeutic purposes.

Studies in mouse models of cancer have demonstrated that the p53 pathway has a pivotal role in restraining Myc oncogenicity, particularly in mesenchymal tumors (e.g., lymphoma[2]), however, additional suppression mechanisms operate in more genetically complex epithelial cancers (e.g., breast cancer[3]). One such suppression mechanism may involve *Bin1*, a gene that encodes nucleocytosolic adapter proteins that can interact and inhibit the transforming activity of Myc.[4,5] *Bin1* has properties of a suppressor or negative modifier gene in cancer: its expression is often attenuated or abolished in breast and prostate cancers, colon cancer, astrocytomas, neuroblastomas, and malignant melanoma, where its ectopic expression can inhibit proliferation and/or promote apoptosis[4,6-12] (Xie K, Prendergast GC, unpublished observations). Notably, the benefit of *Bin1* loss to cell growth and survival appears to be contingent on cell transformation.[13-16] Taken together, these studies suggest that *Bin1* may restrain cellular proliferation and survival in a contextual manner that is dependent on some feature of neoplastic pathophysiology.

While a simple and readily classifiable function has yet to emerge for Bin1 adapter proteins, current information argues that they interact with vesicular membranes and act

as scaffolds to integrate signaling and trafficking processes in cells, including at the nucleus. Bin1 proteins are members of the BAR (Bin/Amphiphysin/RVS) family of adapter proteins that are characterized by a signature fold termed the BAR domain. Structural studies indicate that BAR domains can sense membrane curvature and possibly serve as small GTPase effector modules.[17,18] Some isoforms of Bin1 (a.k.a. Amphiphysin2) associate with endocytotic complexes, however, gene knockout studies in mouse, *Drosophila*, and fission yeast indicate that *Bin1* is nonessential for endocytosis.[16,19-21] Instead, recent studies suggest that this association may reflect some role in trafficking processes.[22,23] In yeast, genetic studies of the conserved BAR adapter proteins (homologs of mammalian *Bin1/Amphiphysin2* and *Bin3*) highlight connections to actin control and stress signaling.[21,24-28] Mammalian cell studies suggest that stress-related BAR adapter functions may extend into the nucleus. For example, Bin1 proteins that can suppress cell transformation localize to the nucleus and can suppress Myc transactivation activity.[5] Similarly, following oxidative stress APPL proteins localize to the nucleus where they mediate growth inhibition, possibly through interactions with the transcriptional repression complex NuRD/Mi-2.[29] Thus, some BAR adapter proteins may link trafficking processes to transcription or other nuclear events in a manner that yet to be fully appreciated. In this study, we exploited a recently developed knockout mouse model[16] to probe the hypothesized anti-proliferative role of *Bin1* in *c-myc*-transformed cells.

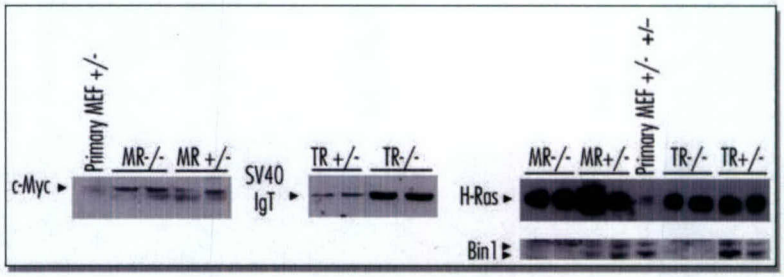

Figure 1. Transgene expression in transformed primary mouse embryo fibroblasts. Cell extracts were generated from the cells indicated and subjected to Western analysis with c-Myc, H-Ras, SV40 large T antigen, and Bin1 antibodies.

MATERIALS AND METHODS

Cell Culture. Mixed 129sv/BL6 mice heterozygous for the *Bin1* gene[16] were mated to heterozygous *p53* null mice (Jackson Laboratories) and offspring from this mating were interbred to generate embryos that were heterozygous or homozygous for each gene. *p53* is a key suppressor of *c-myc*, so to assess the contributions of *Bin1* as a suppressor of *c-myc* we wished to focus solely on the p53-independent effects of *Bin1*. Primary mouse embryo fibroblasts (MEFs) cultured from 13.5 dpc embryos that were $p53^{-/-}$ and either $Bin1^{+/-}$ or $Bin1^{-/-}$ were tranfsormed with oncogenes in vitro as described.[16] Cells with a heterozygous genotype were used to control for the *neo* cassette used for gene replacement at the murine *Bin1* locus.[16] Myc+Ras-transformed MEF populations, referred to as MR MEFs or MR cells, were generated either by expansion of clonal foci produced by transfection of the vectors pLTR-Hm (deregulated human c-*myc*) and pT22 (human c-H-*rasV12*),[30] or by infection of cells with ecotropic retroviruses expressing these genes (see below). SV40 large T+Ras-transformed MEFs, referred to as TR MEFs or TR cells below, were generated by expansion of clonal foci produced by pneoCMV-Tag (SV40 T antigen under the control of the CMV enhancer/promoter) and pT22[30]). TR MEFs have a wild-type p53 genotype, but p53 is inactivated in these cell populations due to interaction with large T antigen. MEFs transformed by the adenovirus E1A region (encoding the 9S, 12S, and 13S E1A oncoproteins) plus activated H-RasV12, referred to as ER MEFs or ER cells, have been described previously.[15] For retroviral gene transduction, the plasmid vector MSCV-pac[33] was modified to express human c-Myc, H-RasV12, or Bin1[-10] or Bin1[-10-13] cDNAs[30,34] via the production of helper-free viruses. To generate retroviruses, 293 cell-derived Phoenix cells[35] plated the previous day at 2 x 10^6 per 10 cm dish were transfected transiently with plasmids mixed with Lipofectamine 2000 as recommended by the vendor (Invitrogen). High titer retrovirus stocks were prepared for use essentially as described.[35,36] To obtain cell populations doubly infected with c-Myc and H-Ras virus, c-Myc retroviral infection was first carried out after which the cells were washed with media and H-Ras retroviral infection was performed. Puromycin at 2 µg/ml was applied 48 hours after infection to remove uninfected cells. Expression of transgenes was confirmed by Western blot analysis. The proliferation and viability of cells cultured on plastic or polyHEMA-coasted tissue culture dishes was performed using sulfarhodamine B or trypan blue exclusion methods as described.[16,37] For flow cytometry, cells were processed as described[38] using a FACscan cell analyzer (Bectin Dickinson).

Western Blot Analysis. Standard methods were used to analyze 50 µg cell extracts prepared in NP40 lysis buffer except for 10 µg cytosolic extracts generated as described for analysis of cytochrome C.[39] Procedures for analysis of Bin1 proteins with monoclonal antibody 2F11 have been described.[40] Methods recommended by the vendors were used to analyze c-Myc, H-Ras, TRADD, caspase-3, and caspase-9 (Santa Cruz Biochemicals); IκB, phospho-IκB, and Bid (Cell Signaling); PARP (Chemicon); and cytochrome C (Pharmingen).

Endocytosis Assay. Endocytic uptake of FITC-conjugated transferrin (Molecular Probes) was assessed in MR MEFs by flow cytometry 30 min after addition to cells in growth media as described previously.[16]

EMSA Assay. Electrophoretic mobility shift assay for NF-κB was conducted using nuclear extracts prepared 4 hr after treatment of ER MEFs with 100 ng/ml TNF-α, essentially as described.[41] The oligonucleotide used for the probe encoded the sequence CATGAGTTGAGGGGACTTTC-CCAGGC.

Tumor Formation Assay. 1 x 10^6 cells suspended in 200 µl of DMEM were injected into F1 syngeneic hosts produced by crossing 129S1/SvImJ and C57BL/6J breeders (Jackson Laboratories). Tumor growth was monitored by caliper measurements. Necropsy was performed at the two week endpoint and final tumor measurements and wet weight were recorded. Tumor volumes were calculated using the formula width^2 x length x 0.52. Statistical analysis was performed using Prism4 software.

RESULTS

***Bin1* Deletion Promotes Growth and Tumor Formation in *c-myc+ras*-Transformed Cells.** Transformation of primary rodent cells by *c-myc* requires a collaborating oncogene such as activated *ras*. Therefore, to examine the effects of *Bin1* deletion on *c-myc* oncogenicity we compared the phenotype of primary mouse embryo fibroblasts (MEFs) that were cotransformed with plasmids encoding human *c-myc* and activated *H-rasV12*, termed below MR MEFs or MR cells. Our experience with MEFs is that they are refractory to transformation by *c-myc+ras* if the p53 pathway is intact, so MR MEFs generated in this study were derived from a $p53^{-/-}$ background (i.e., $Bin1^{+/-}$ $p53^{-/-}$ versus $Bin1^{-/-}$ $p53^{-/-}$ MR MEFs were compared; heterozygous cells were used to control for the neo cassette used for gene replacement at the *Bin1* locus[16]). This design was also desirable because it allowed an assessment of the p53-independent effects of *Bin1* suggested by previous experiments.[5,13] We also generated additional transformed MEF populations on a wild-type *p53* gbackground using the SV40 large T antigen plus activated *H-ras* (termed TR MEFs or TR cells). Transformed MEF populations were derived from individual cell foci that arose from monolayer cultures of primary cells after oncogene transfection and also, in the case of MR MEFs, from cells infected with ecotropic recombinant retroviruses. We observed increased

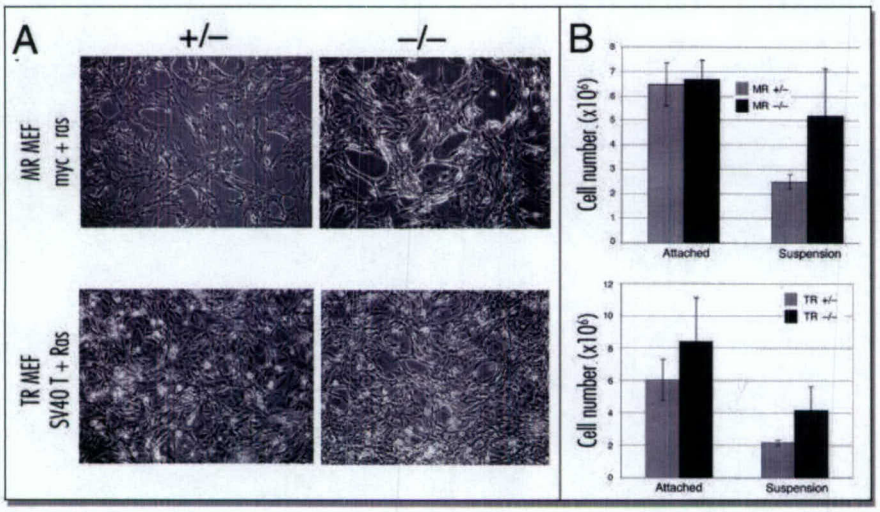

Figure 2. Effect of *Bin1* loss on cell morphology and anchorage-independent proliferation. (A) Morphology. Cells were photographed at 100x magnification using phase optics. (B) Cell proliferation. Standard SRB assay was conducted on plastic tissue culture dishes or dishes coated with the nonadherent substratum polyHEMA as described.[37] 1 x 10^6 cells were seeded into growth media in triplicate and counted 72 hr later. The experiment was performed twice.

efficiency of MEF focus formation associated with Bin1 loss (see Fig. 1S), however, this effect varied with different serum lots, causing us to focus instead on the effects of *Bin1* loss in stably transformed cells. Multiple cell populations were used to rule out possible clonal artifacts as well as address cell-type issues. In all the cell populations used, the *Bin1* ggenotype was confirmed by PCR and Western blot analysis, and transgene expression was confirmed by Western blot analysis (Fig. 1).

***Bin1* Loss Accentuated the Transformed Phenotype and Growth of MR MEFs.** Under normal growth conditions, *Bin1*$^{-/-}$ cells were more refractile and spindle-like compared to their Bin1-expressing counterparts (Fig. 2A). Anchorage-dependent proliferation of attached cells was not significantly affected by loss of *Bin1*. In contrast, *Bin1* loss conferred a significant advantage to growth under anchorage-independent conditions (forced by culturing cells on the nonadherent substrate polyHEMA) (Fig. 2B). Flow cytometry showed no difference in the apoptotic susceptibility of *Bin1*$^{-/-}$ cells under anchorage-independent conditions (data not shown), consistent with evidence that p53 disruption and *ras* gactivation greatly reduce sensitivity to *c-myc*-mediated apoptosis.[1] Thus, we attribute the superior outgrowth of the *Bin1*$^{-/-}$ *p53*$^{-/-}$ MR MEFs under these conditions to enhanced proliferation. *Bin1* loss conferred a similar anchorage-independent growth advantage to TR MEFs (Fig. 2B). However, this effect could be derivative of a benefit to c-*Myc*-mediated proliferation, because T antigen requires *c-myc* activity to drive cell proliferation.[42] The growth inhibitory effects of *Bin1* could not be mediated by the p53 or Rb pathways, because p53 was inactivated by targeted gene deletion or T antigen binding in the MR or TR cells, respectively, and because Rb was suppressed by Myc activity or T antigen binding in the MR or TR MEFs, respectively.[43,44] We concluded that *Bin1* restrains anchorage-independent cell proliferation through a p53/Rb-independent mechanism.

Tumor formation experiments showed that *Bin1* loss similarly enhanced the transformed growth of MR MEFs in vivo. At a two week endpoint, the mass of tumors formed in syngeneic hosts after s.c. injection of MR MEFs was markedly greater for cells that lacked *Bin1*, consistent with the effects of *Bin1* loss on in vitro proliferation (Fig. 3A). The negative impact of Bin1 expression on tumor growth was less pronounced in nude mouse hosts (data not shown), consistent with the finding that Bin1 loss can contribute to tumorigenicity in a cell extrinsic manner (Muller et al, manuscript in preparation). Interestingly, the benefit of *Bin1* loss to tumor formation was not replicated in TR MEFs, and indeed, loss of *Bin1* markedly limited tumor outgrowth in syngeneic mice (Fig. 3A). This observation indicates that the effects of *Bin1* loss on *c-myc*-mediated tumor formation are specific. In addition, it suggests that *Bin1* loss may confer an in vivo benefit to *c-myc*-mediated tumor formation beyond anchorage-independent proliferation, because TR MEFs gained

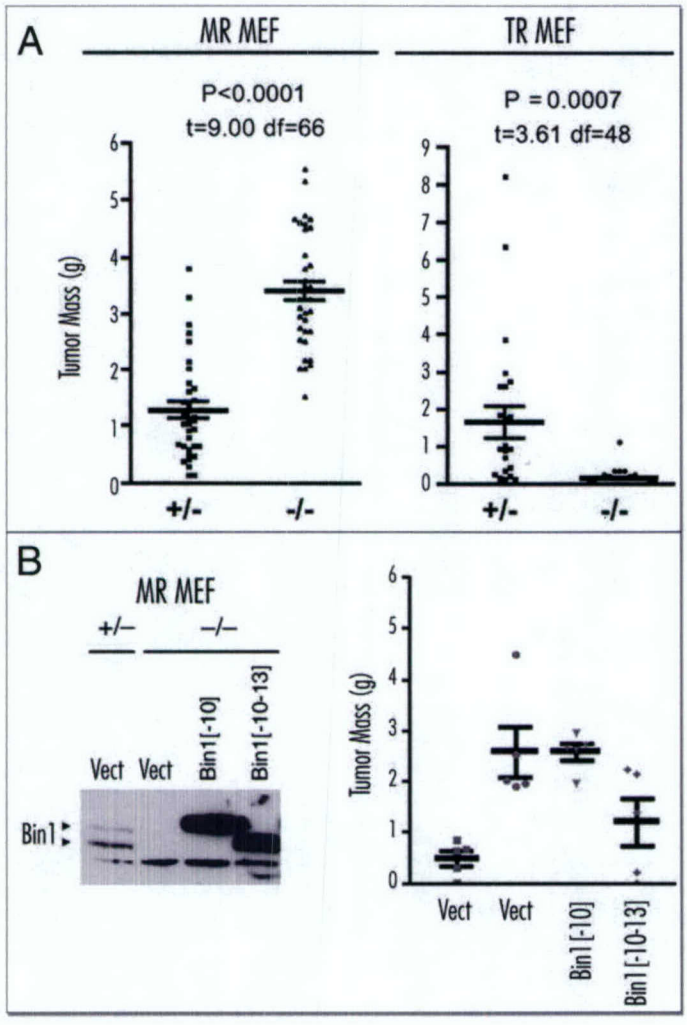

Figure 3. Effect of *Bin1* loss on graft tumor formation. (A) Selective benefit of *Bin1* loss to tumor formation by *c-myc+ras*-transformed cells. 1 x 10^6 MR MEFs or TR MEFs were injected s.c. into syngeneic F1 129sv x BL/J6 mice and tumors were scored two weeks later. Each point on the graph represents a single tumor measurement. The mean and standard error of the data are shown. Unpaired P value was computed from two-tailed T values (t = t ratio [difference between sample means divided by the standard error of the difference between the means]; df = degrees of freedom [n - 2]). (B) Complementation assay. *Bin1*$^{-/-}$ MR MEFs were infected with recombinant retroviruses expressing no insert or one of the two ubiquitous Bin1 isoforms (Bin1-10 or Bin1-10-13). Western blot analysis confirmed transgene expression (panel). Cells were injected and tumors were scored as above.

the latter without better tumor growth. Although the nature of this in vivo effect was not defined here, the benefit to tumor growth conferred by *Bin1* loss could be reversed in MR MEFs by retroviral-mediated complementation with Bin1[-10-13],[45] one of the two ubiquitous splice isoforms of the gene (Fig. 4B). This finding further validates the capacity of *Bin1* to restrain in vivo tumorigenicity. We concluded that *Bin1* restrains the transformed rowth of *myc+ras*-transformed fibroblasts in vitro and in vivo, possibly by distinct mechanisms.

***Bin1* Loss Does not Abrogate Endocytosis but Increases Sensitivity to Paclitaxel.** Overexpression of the SH3 domain from Bin1/Amphiphysin2 proteins inhibits endocytosis[46] and this result has been interpreted as evidence of a essential role for *Bin1* in endocytosis. Impairing endocytosis could conceivably promote cell growth by increasing the time that growth factor receptors could signal from the cell surface. Recently, knockout studies in fission yeast, fruit flies, and mice have demonstrated that *Bin1* is nonessential for endocytosis.[16,19-21] Nevertheless, because homologs of *Bin1* in yeast have been implicated in stress signaling,[21,24] we wished to examine the possibility that *Bin1* may be essential to support endocytosis under conditions of transformation-associated stress. However, the endocytotic capability of MR MEFs was unaffected by *Bin1* loss as assessed by uptake of FITC-labeled transferrin (Fig. 4A), as was observed previously in untransformed primary cells.[16] An additional finding that may relate to this issue was that *Bin1* loss increased the sensitivity of cells to paclitaxel (Fig. 4B), a drug that disrupts microtubule dynamics required for endocytotic trafficking.[47] Clonogenic growth assays confirmed that *Bin1* loss conferred sensitivity under conditions of long-term drug treatment (Fig. 5B). We concluded that the effects of *Bin1* loss in MR MEFs produces an elevated sensitivity to microtubule disruption but not a generalized defect in endocytosis.

***Bin1* Loss Limits the Susceptibility of E1A+ras-Transformed Cells to Tumor Necrosis Factor-α.** Several studies have shown that *Bin1* can facilitate programmed cell death[6,10,12,14,15] but its possible effects on extrinsic mechanisms of apoptosis mediated by death receptor ligands such as tumor necrosis factor-α (TNF) have not been examined. Unfortunately, the p53 null MR MEFs generated in this study were not susceptible to TNF-induced cell death. Therefore, to examine this question we used MEFs transformed by the adenovirus E1A early region plus activated *H-ras* (termed ER MEFs or ER cells) that had been generated for another study.[15] This relevance of this system is justified by functional similarity between *c-myc* and E1A in murine cell transformation,[48] the requirement of *c-myc* for E1A to drive cell proliferation,[42] and the well-documented ability of the E1A early region to sensitize murine fibroblasts to TNF-induced apoptosis (e.g., ref. 49). Previous work showed that as in the case of TR MEFs *Bin1* loss does not promote tumor formation by ER MEFs.[15]

We observed that *Bin1* deletion in these cells ablated sensitivity to TNF-induced apoptosis (Fig. 5A). Western analysis of a set of key TNF signaling proteins showed similar expression of the TNF receptor-binding adapter protein TRADD and similar regulation of the NF-κB regulatory factor IκB in each cell population, however, downstream caspases and effector events including cleavage of PARP and Bid and mitochondrial release of cytochrome C were activated only in the $Bin1^{+/+}$ cells (Fig. 5B and 5C). NF-κB activity is a key determinant of the TNF response. In electrophoretic mobility shift assays, we found that TNF stimulated NF-κB activity in both cell populations. However, $Bin1^{-/-}$ cells differed in their exhibition of a low basal level of precocious nuclear NF-κB activity (Fig. 5D). The stimulation of NF-κB in both settings was consistent with the lack of a defect in IκB regulation, although it begged the question of how basal NF-κB activity was increased in null cells. Nevertheless, since elevated NF-κB limits TNF-induced cell death, we confirmed the expectation that cycloheximide (CX) cotreatment could restore TNF sensitivity by ablating NF-κB (Fig. 6). CX is used to sensitize primary MEFs to TNF. Earlier studies suggested that *Bin1* was more likely to be involved in E1A-mediated sensitization to TNF rather than to be required for TNF-induced cell death. This expectation was met by the lack of an effect of *Bin1* deletion on the ability of TNF+CX to kill primary MEFs (Fig. 6). In summary, the results support the concept of a modifier role for *Bin1* in shaping the response of ER cells to an extrinsic cell death signal.

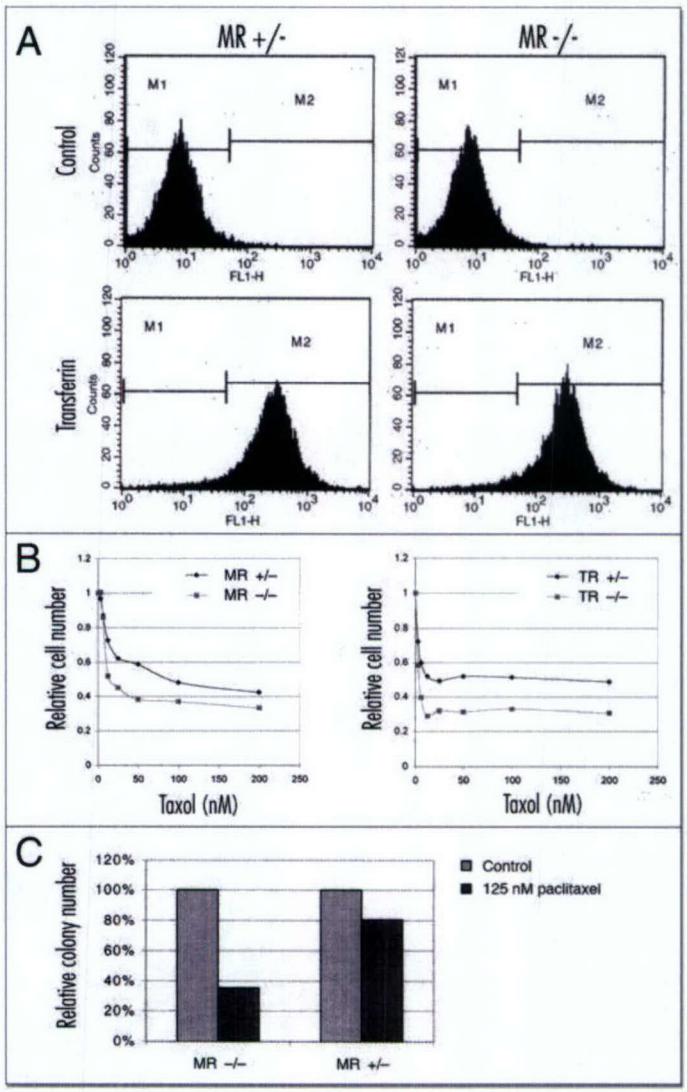

Figure 4. *Bin1* loss does not abrogate endocytosis but sensitizes cells to paclitaxel. (A) Receptor-mediated endocytosis. Uptake of FITC-conjugated transferrin was documented by flow cytometry 30 min after addititon to MR MEF cultures as described.[16,62] (B) Growth assay. SRB assay was performed 72 hr after exposure of cells in growth media to the concentration of paclitaxel indicated. (C) Clonogenic assay. Relative colony formation was determined by crystal violet staining 13 days after treatment with 125 nM paclitaxel of 4000 cells seeded overnight into a 100 mm dish.

DISCUSSION

This study offers the first direct evidence that the *Bin1/Amphiphysin2* gene acts to restrain neoplastic cell growth. Previous studies have found that *Bin1* expression is diminished in many cancers and that ectopic overexpression of nuclear-localizing Bin1 splice isoforms limits the proliferation or survival of malignant cells. Here the use of nullizygous mouse cells[16] allowed the direct demonstration that *Bin1* suppresses the anchorage-independent growth, tumorigenicity, and apoptotic susceptibility of neoplastically transformed cells.

This work addresses an hypothesized role for *Bin1* in restraining the oncogenicity of *c-myc*, focusing on p53-independent mechanisms of restraint. This focus is justified by a need to address existing gaps in knowledge concerning the identity of functions that can

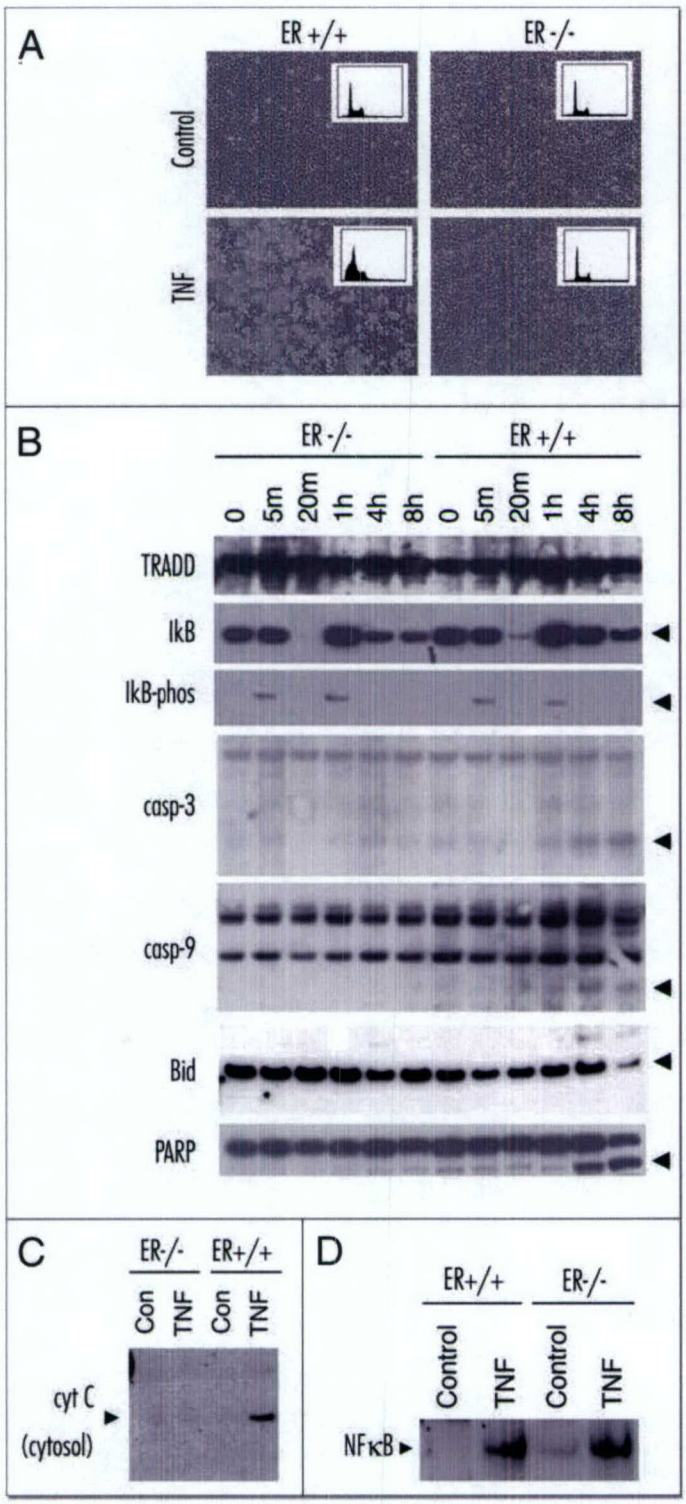

Figure 5. Effect of *Bin1* on TNF-induced cell death in E1A+ras-transformed cells is associated with altered NF-κB activity. (A) Morphology and flow cytometry. ER MEFs in growth media were incubated 24 hr with 100 ng/ml TNF-α before being photomicrography and processing for flow cytometry (insets). (B) Western analyis. Cell extracts were prepared at the times indicated following exposure to 100 ng/ml TNF-α before processing for Western blotting. (C). Cytochrome c release. Cell extracts were prepared and cytosolic fractions were processed for Western blotting 8 hr after cell treatment with 100 ng/ml TNF-α.

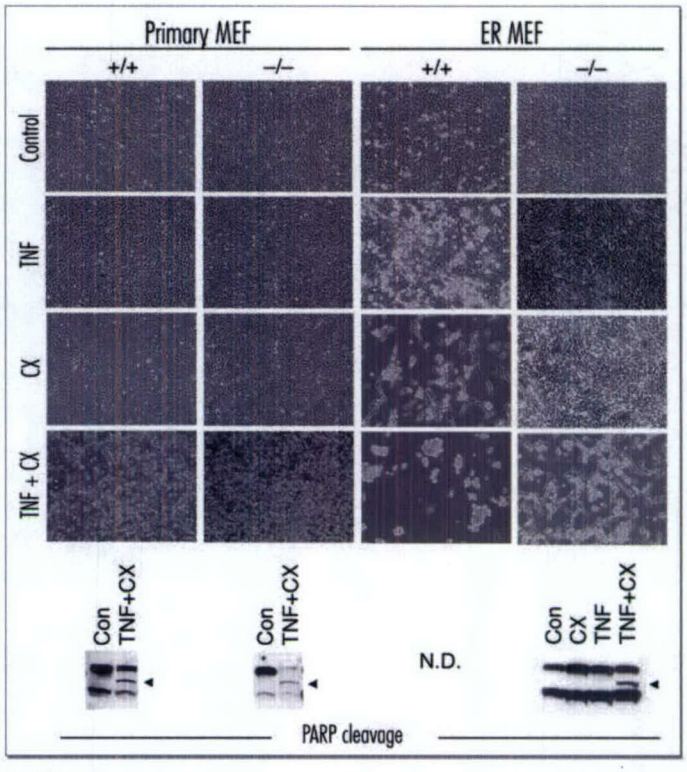

Figure 6. Transformation-selective effect of *Bin1* on the TNF response. Primary MEFs or ER MEFs with the genotypes indicated were treated 24 hr in growth media with 100 ng/ml TNF-α, 5 μg/ml cycloheximide (CX), or both agents before photomicrography and processing for Western blotting of the caspase-3 substrate PARP.

restrain g*c-myc* gwithout the apparent involvement of the p53 pathway.[50-54] *Bin1* loss promoted anchorage-independent growth in p53 null MR MEFs and in TR MEFs (where p53 is inactivated by T antigen binding), demonstrating that *Bin1* can indeed restrain *c-myc*-mediated growth in transformed cells in a p53-independent manner. This effect did not occur under conditions of anchorage-dependent growth. Since *Bin1* acts only in the absence of substratum attachment, one might expect its role in cancer to be confined to cells that have acquired the ability to proliferate in the absence of a physiological extracellular matrix (ECM). The findings of this study are consistent with other evidence that *Bin1* does not influence cell growth or survival in primary rodent or avian cells or in nonmalignant human cells.[13,14,16] The evidence that the inhibitory effects of *Bin1* are selective for a defining feature of cancer pathophysiology (anchorage-independent growth) argues strongly for a negative modifier role rather than a generalized suppressor role (i.e., a gene that can restrain cell proliferation generally during neoplastic development, e.g., Rb).

The results of tumor formation experiments offer additional support for the conclusion that *Bin1* negatively modifies the oncogenicity of *c-myc* in cancer. While both MR and TR cells benefitted from *Bin1* loss in terms of anchorage-independent growth, only MR cells benefitted in terms of tumor formation. *Bin1* loss did not benefit tumor formation by TR MEFs (this study) or ER MEFs.[15] Thus, it is evident that *Bin1* has a selective effect in the context of oncogenic transformation mediated by *c-myc* that is not shared with the viral oncoproteins, despite their parallel roles in cell transformation. Such selectivity is a hallmark of modifier functions. The dichotomy

between in vitro and in vivo effects hints further at interactions with the stromal microenvironment, a possibility consistent with the observation that substratum adhesion status dictated the impact of *Bin1* on cell growth. Consistent with this idea, we have determined that *Bin1* loss can promote escape from host anti-tumor immunity (Muller et al., manuscript in preparation). This benefit may contribute in part to the enhanced tumorigenicity of Bin1-null MR MEFS, as Bin1-expressing MR MEFs were found to be relatively more tumorigenic in immunodeficient nude mice than in immunocompetent syngeneic animals.

This study assessed possible roles for Bin1 proteins in endocytosis, based on the presence of some splice isoforms (Amphiphysin2 isoforms) in endocytotic complexes and on the ability of BAR adapter proteins generally to interact with curved vesicular membranes.[17,46] Genetic experiments in mice, fruit flies, and fission yeast, have established that *Bin1* homologs are nonessential for endocytosis, however, evidence from yeast of an essential role in stress responses[21,24] prompted us to address whether *Bin1* may support endocytosis under conditions of transformation stress. While no generalized effects on receptor-mediated endocytosis were seen, an increased sensitivity of *Bin1*-null cells to paclitaxel, a drug that disrupts microtubule dynamics, was noted. This observation may be interesting in three regards. First, microtubule dynamics are essential for vesicle trafficking[47] and Bin1/Amphiphysin2 proteins have been implicated recently in vesicle trafficking processes.[23] Second, there is significant clinical interest in molecular markers of taxane sensitivity, which remain largely unknown.[55] Lastly, in light of the effects of *Bin1* on *c-myc*-mediated oncogenicity, the effects of *Bin1* on paclitaxel sensitivity are intriguing given the well-documented interaction of c-Myc proteins with α-tubulin in tumor cells.[56,57]

The last aspect of this work examined a role for *Bin1* in TNF-induced apoptosis. Earlier studies argued that *Bin1* can facilitate programmed cell death in certain transformed cell or cancer cell settings, in some cases via intrinsic mechanisms, but potential intersections with extrinsic mechanisms of cell death had not been examined.[7,8,13-15] Due to the unresponsive nature of the MR MEFs generated, probably due to the suppressive effects of p53 deletion and g*ras* gactivation, we were unable to determine whether *Bin1* may influence the ability of *c-myc* to sensitize these transformed mouse cells to TNF-induced apoptosis.[58-60] However, using ER MEFs that were susceptible to TNF, we observed that *Bin1* loss abrogated E1A-mediated sensitization to TNF. As above, the role of *Bin1* was germane only in the transformed cell setting. Along with other evidence that *Bin1* acts in a context-dependent manner, these findings tended to reinforce the major conclusion of this study that *Bin1* acts as modifier function in cancer. Mechanistic investigation suggests that *Bin1* may influence NF-κB control at some level, although the basis for such influence is obscure. Still, two recent reports may encourage further examination of this direction. First, interaction has been reported between the *Drosophila* orthologs of Bin1 and MyD88 (Tube), one of the canonical regulators of the NFκB (Dorsal) pathway in flies.[61] Second, there is a precedent for the concept that BAR domain functions are important for appropriate trafficking of a transcription factor in STAT3.[22] Further work is needed to gauge the breadth or impact that *Bin1* may have on the efficiency of NF-κB trafficking in transformed cell settings.

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AUTHOR: PLEASE COMPLETE REFERENCES BY LISTING ALL AUTHORS

Variant loxP site facilitates construction of embryonic stem cell lines for conditional knockout mice

Meeyoung Chang[1], Wei Weng[2], George C. Prendergast[1,3], and Alexander J. Muller[1]

[1]Lankenau Institute for Medical Research, Wynnewood PA 19096 USA; [2]Ingenious Targeting Laboratory, Inc., Stony Brook NY 11790 USA; and [3]Department of Pathology, Anatomy & Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia PA 19106 USA

Running title: Utility of variant loxP site for mouse gene targeting

Corresponding author: Alexander J. Muller, Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood PA 19096. Phone 610.645.8034. Fax 610.645.8091. Email mullera@mlhs.org

Abstract

Cre-lox technology allows the production of conditional gene deletions, however, obtaining a suitable embryonic stem (ES) cell line to make chimeric mice can be challenging. Generally the targeting vector includes a loxP- selectable marker-loxP-target-loxP cassette ('tri-lox' cassette) flanked by DNA sequences that mediate homologous recombination into the ES cell genome. In successfully targeted ES cell clones, Cre recombinase is expressed transiently with the aim of excising the selectable marker but not the target sequences. A common barrier at this stage is difficulty in obtaining the subclone due to excision of the entire cassette by Cre. Here we show that including a point mutation in the 3'-most loxP site in the targeting vector facilitates selective excision of the marker without abrogating later deletion of the targeted sequence in vivo. Various applications of this strategy including for generating genetic mosaics are discussed.

Introduction

Genomic manipulation via homologous recombination provides a valuable tool to explore gene function(2). In particular, the use of cre-lox technology offers a powerful method to generate conditional loss of function or change of function alleles in mice(8). This technology employs the Cre recombinase encoded by bacteriophage P1, a member of a large family of bacterial DNA recombinases that includes Flp and Int. Cre recognizes a specific 34-bp DNA element termed loxP from which it catalyzes the excision of DNA sequences via formation of a covalent protein-DNA complex and a Holliday junction intermediate(7). In animal cells, Cre can be exploited to mediate excision of DNA sequences located between different loxP sites in a highly precise manner. Specifically, embryonic stem (ES) cell clones can be created in which a dominant selectable marker gene and a desired DNA sequence that are flanked by loxP sites (floxed) are inserted into the ES cell genome by homologous recombination. In the simplest design, the floxed allele has the structure gene–loxP–selectable marker–target–loxP–gene. However, while ES cell clones with this configuration can be used to generate chimeric animals, one potential complication is that the presence of the selectable marker gene may disrupt appropriate regulation of the adjacent targeted gene at times before Cre-mediated excision is initiated. Therefore, to avoid this complication, many investigators vary the structure of the targeting construct by inserting an additional loxP site between the marker and target, thereby allowing for removal of the marker in the cassette by partial Cre-mediated recombination(5). For this strategy, Cre is expressed transiently, typically by electroporation of a plasmid vector, and then ES cell subclones with the desired genotype are identified. One frequent problem at this stage results from the fact that in most cells Cre excises the entire cassette, including the target sequences. Indeed, in many cases, the desired partial

recombination event occurs rarely or not at all, preventing the ability to obtain the desired ES cell clone for chimera generation. While it is possible to combine a FLP-based strategy to selectively delete the selectable marker with a Cre-based strategy to delete the target sequences (using an appropriately designed targeting vector), FLP works less efficiently than Cre both *in vitro* and *in vivo*(15), increasing the complexity and effort involved to identify suitable ES clones and suitable germline chimeric mouse strains.

In this report, we illustrate a simple and efficient strategy to overcome this common barrier to generating 'flox' knockout mice, based on introducing a point mutation into the 3'-most loxP site of a standard 'tri-lox' targeting vector. We show that this strategy confers a selective advantage for achieving Cre-mediated excision of the marker *in vitro* without abolishing the subsequent ability of Cre to delete the remaining floxed target sequence *in vivo*. Mice generated in this way can be effectively utilized for standard, targeted gene disruption by tissue-specific expression of Cre. This strategy also provides for a more efficient way to generate mosaic animals where recombination is distributed throughout the animal. Advantages of mosaic mice (as compared to the commonly used tissue-restricted knockout mice) are discussed for evaluating questions of how gene loss impacts tumorigenesis.

Results

We used standard methods to generate a conditionally targeted ('floxed') allele of the murine *Bin1* gene, except that a variant loxP site was incorporated at the most distal position in the targeting vector. Briefly, the targeting vector was constructed in which a neomycin resistance gene (neo)

cassette flanked by wild-type loxP sites was inserted into 129sv genomic DNA spanning introns 2-5 of the mouse *Bin1* gene(9) (Fig. 1). Approximately 1 kb downstream of the neo casette a variant loxP site incorporating a T->C transition at position 7 was inserted, such that exon 3 was flanked on its 5' side by a wild-type loxP site and on its 3' side by the variant loxP site (Fig. 1). This point mutation is reported to decrease the binding affinity of one Cre recombinase monomer for the loxP half site by ~30-fold(4). ES cell clones produced by electroporation of the targeting plasmid and G418 selection were screened for homologous recombination by PCR using primers located within the neo gene and the flanking regions (as indicated by thin arrows in Figure 1). In one clone, Cre was expressed transiently by electroporation of the expression vector pOG231(11, 12). PCR analysis of 30 colonies generated by limiting dilution of the electroporated cells was performed. Eight colonies exhibited deletion of the neomycin marker cassette and 3/8 of these colonies (38%) retained the adjacent floxed exon 3 sequences (data not shown). In our experience, this frequency of partial recombination was considerably more efficient than that produced in ES cell clones where the loxP sites used in the targeting construct each have the wild-type sequence. For example, in a separate project where only wild-type loxP sites were used, the analysis of 48 ES cell colonies analyzed after transient expression of Cre showed excision of the entire cassette in all of the colonies analyzed (W.W., unpublished observations). We concluded that inclusion of the 3' variant loxP site in the *Bin1* targeting vector reduced the efficiency of Cre-mediated recombination at that site, thereby favoring recombination at the two 5'proximal sites to faciliate selective deletion of the neo marker.

Subsequent analysis of mice generated from the targeted ES cells demonstrated that the variant loxP site was able to support Cre-mediated recombination *in vivo*. Briefly, standard methods were

used for ES cell injection into blastocysts and implantation resulting in the production of two germline chimeric mice (data not shown). Each mouse harbored the desired floxed exon 3 allele (Fig. 1), the structure of which was confirmed by PCR and DNA sequencing (data not shown). Tissue-specific excision was confirmed by examining the structure of the floxed Bin1 allele in female offspring generated from crosses to WAP-Cre transgenic animals. The whey acidic protein (Wap) promoter is used commonly to target gene expression specifically to the mammary gland epithelium(16). Recombination in Wap-Cre transgenic mice is largely restricted to the mammary gland with occasional recombination occurring in the brain(16). Homogeneous high level expression from the Wap promoter is confined to the lactational stage of pregnancy, although transient expression is also observed during the estrus cycle(14). In the conditional flox *Bin1* knockout mice created, PCR analysis revealed no evidence of recombination in the mammary gland of virgin females (data not shown), where the wap promoter is inactive, but evidence of recombination in the mammary gland of nursing females where activation of the wap promoter drives Cre expression (Fig. 2A). Recombination in mammary gland tissue was detected from late pregnancy through weaning, although by full regression stage after weaning the product of the recombined allele was faint due to loss of epithelial cells caused by involution (Fig. 2B). In summary, use of the variant loxP site facilitates the ability to obtain an ES cell population with the desired flox allele without abrogating the *in vivo* competency of the allele to be targeted in a tissue-specific manner by Cre recombinase.

Systemic deletion produces an embryonic or perinatal lethal phenotype for many genes including *Bin1*(10). The perinatal lethal phenotype produced by *Bin1* deletion offererd a direct genetic test to verify that that the recombined *Bin1*(floxΔ) allele was functionally inactive. To demonstrate that

the *Bin1*(floxΔ) phenocopies the *Bin1*-null allele, termed here *Bin1*(KO), we introduced the recombined *Bin1* floxΔ allele into the germline by crossing the *Bin1*(flox/flox) transgenic mouse with the transgenic EIIa-*Cre* mouse line in which *Cre* expression is controlled by the EIIa promoter(6). In the absence of adenovirus E1A coactivator, expression driven by the EIIa promoter is restricted to mouse oocytes and preimplantation embryos including the 1-cell stage zygote.(3) The EIIa-*Cre* transgenic mouse is therefore useful to obtain mice with complete recombination of loxP alleles in all cells in the mouse including the germline. Heterozygous *Bin1*(floxΔ/wt) germline recombinants were interbred with *Bin1*(KO/wt) mice and five independent litters were analyzed. Of the neonates obtained, 11/52 (21%) were clearly unhealthy at birth and died shortly postpartum. These pups were all *Bin1*(floxΔ/KO) and accounted for all the neonates in the five litters with this genotype. Histological analysis of the hearts of dead pups confirmed the presence of severe ventricular hypertrophic cardiomyopathy in the Bin1(floxΔ/KO) mice (Fig. 3), as documented previously in Bin1 (KO/KO) neonates(10). On the basis of these results, we concluded that the fully recombined *Bin1*(floxΔ) allele was biologically inactive.

When EIIa-Cre mediated recombination occurs later than the 1-cell zygote stage the result is a mosaic animal in which only a subset of cells harbor the recombined allele. Producing a mosaic knockout mice may therefore provide a strategy to bypass developmental lethal phenotypes associated with complete gene knockout (like the case with *Bin1* knockout). EIIa-Cre-mediated recombination of loxP-flanked alleles has been reported to produce complete systemic deletion in 50% of the progeny produced with the remaining 50% of the progeny being mosaics(6). Using the variant 3' loxP site, we observed a frequency of 6% complete systemic recombinants, 60% mosaic animals, and 34% non-recombinants (Table I and Fig. 4). These results provide a measure of the

competency of the flox allele to be targeted by Cre *in vivo*. We also found observed that, while the frequency of recombination varied between individual mosaic mice, as would be expected, the proportion of recombined to non-recombined alleles was consistent across all tissues examined from each mouse (Fig. 5). We concluded that use of the variant 3' loxP site did not compromise *in vivo* recombination and was useful for more efficient production of mosaic animals.

Discussion

This report describes a simple strategy to promote partial Cre-mediated recombination within a 'tri-lox' allele to achieve specific excision of a floxed DNA region. This method addresses a common barrier to the production of conditional knockout mice, where one wishes to remove a dominant selectable marker from a gene targeting construct that has been integrated homologously into the genome of an ES cell line. This strategy, which is generalizable to any 'tri-lox' design where an intermediate recombination step is desired, is based in an apparent difference in the efficiency by which Cre recognizes and/or mediates recombination from a mutated loxP site *in vitro* versus *in vivo*. While it is possible to design strategies using both FLP and Cre recombinases to achieve distinct step-wise deletions in a targeting construct(15), the less efficient activity of FLP recombinase both *in vitro* and *in vivo* can increase the complexity and labor required to identify suitable ES cell clones and/or mouse strains. By comparison, the strategy defined here facilitates generation of the desired ES cell clone in a simple and labor-efficient manner.

The use of altered loxP sites offers a new tool to vary the use of Cre-lox technology in mice. By choosing sites with different affinities for Cre, it is possible to vary the efficiency of gene

knockout. One way the utility of such variation is demonstrated through its application to the production of mosaic animals. Reducing the efficiency of recombination between the loxP sites increased the total proportion of mosaic animals produced and lowered the overall proportion of recombined cells within the mosaic population. Within each individual mouse, however, the proportion of cells harboring recombined vs. non-recombined alleles was shown to remain constant throughout different tissues. Thus, the proportion of recombined to non-recombined alleles can be predicted for internal organs through non-invasive analysis of a standard tail biopsy, and experimental cohorts can be preselected based on the expected level of recombination. The method offered complements other *in vivo* methods for floxed cassette removal that have been described(17), allowing investigators to take fuller advantage of the common tactic of using adenoviral-Cre vectors to generate desired ES cell lines *in vitro.*

Analysis of mosaics as a means to study otherwise lethal mutations has strong precedence in Drosophila(13), but this approach is not common in the field of mouse genetics even though it can be used successfully to rescue embryonic lethal phenotypes(1). Here, the possibility that only a very low proportion of recombined cells (*Bin1* null cells) would be developmentally tolerated proved to be unfounded. Complete systemic disruption of the *Bin1* gene results in complete perinatal lethality that is associated with severe ventricular hypertrophic cardiomyopathy(10). Mosaic *Bin1*-null animals, however, have survived to adulthood, even in cases where there is a high proportion of recombined alleles in all organs including the heart. This unexpected result indicates that a small proportion of wild type cells is sufficient to compensate for the developmental defect associated with *Bin1* loss. Depending on how common this situation may be for other genes, the use of mosaics may allow one to refine understanding of roles in normal

development and physiology or disease pathophysiology. Such insights may be quite valuable, for example, to gauge how varying gene function may differentially affect normal processes versus disease processes in a tissue where therapeutic inventions are desirable.

Mosaic models offer a number of inherent advantages for disease research. In cancer studies, for example, where one wishes to investigate how disrupting a putative tumor suppressor or negative modifier gene like *Bin1* can impact tumorigenesis, mosaic animals offer a number of potential advantages over the more commonly used approach of directly targeting gene with a tissue specific-specific *Cre* gene. First, null cells are distributed throughout every tissue, so the impact of gene loss in different organs can be evaluated without having to generate multiple independent lines harboring different tissue-specific Cre-expressing alleles. This permits an unbiased survey to be conducted to assess the impact of gene loss on tumor development in tissues throughout the body. Second, each mouse is internally controlled (provided that the gene of interest is haplosufficient), because tissues have both null (recombined) and expressing (non-recombined) cells. Assuming a mean representation of 50% recombined and 50% non-recombined alleles, the use of mosaic animals can double the power of the analysis for each mouse that is evaluated. Thus the number of mice needed to obtain statistically relevant data can be significantly reduced. Selecting a population of mice with a lower proportion of recombined alleles will make the analysis more stringent, while selecting for a higher proportion will make it less so. Since the proportion of recombined to non-recombined alleles can be predicted for internal organs from PCR analysis of a standard tail biopsy, identifying the desired population to study is technically straightforward. Third, mosaic analysis is particularly useful in a mixed genetic background, as the paired control is derived from the same animal rather than from a different littermate with a

different distribution of parental alleles. Fourth, Cre expression is restricted to very early embryogenesis, alleviating the concern that contemporaneous Cre recombinase activity may influence the phenotype (a common issue in tissue-specific knockouts). Lastly, the mosaic model allows one to gauge the impact of field effects by allowing one to vary the extent of gene loss in a tissue. Standard tissue-targeted recombination usually results in the gene of interest being ablated in most or all the targeted cells. For cancer, as well as many other diseases, this situation is quite different from the typical environmental context where a tumor arises, in which a clonal cell population with the acquired mutation is typically surrounded by non-mutated cells. With a mosaic model, it is possible to restrict the study to a population of mice in which the proportion of recombined alleles is low and is more reflective of the host environment. On the other hand, if a high proportion of target cells is desired, a population of mice with a high proportion of recombined alleles can be studied (assuming that they are viable).

The development of conditional mouse knockout mice has become an essential technology for basic research aimed at determining gene function in the *in vivo* context as well as for pre-clinical target validation studies. This study provides a strategy to overcome a major technical challenge to the production of conditional knockout mice, and it shows how this strategy can be applied not only to the production of standard tissue-targeted knockouts, but also to facilitating the production of mosaic knockouts, which have a number of distinct advantages for studies of tumorigenesis and other pathophysiological processes.

Materials and Methods

Production of embryonic stem cell lines and germline chimeric mice. Using standard methods, a targeting plasmid with the structure presented in Figure 1 was electroporated into embryonic stem (ES) cells derived from 129sv mice and cell clones were identified by G418 selection. Three clones which integrated the plasmid by homologous recombination were defined by PCR analysis and the structure of the desired targeting event was confirmed by DNA sequencing. These ES cell lines were subsequently electroporated with the Cre vector pOG231(11, 12) and subcloned by limiting dilution to identify colonies in which selective excision of the neo marker had occurred. Of 30 colonies analyzed 8/30 sustained deletion of the neo marker gene and 3/8 of the recombined clones retained the floxed exon 3 segment (Fig. 1). Two ES subclones were microinjected into C57BL/6J blastocysts and two highly chimeric animals exhibiting germline transmission of the floxed *Bin1* allele were generated. To establish a simplified binary system of either Bin1-expressing or non-expressing cells, the conditional Bin1 floxed allele (flox) was placed through genetic crosses over the constitutive Bin1 knockout allele (KO) that had been created and characterized previously(10). Cre alleles to mediate the excision of exon 3 to generate fully recombined offspring (floxΔ) were introduced through crosses originating with the two transgenic strains FVB-TgN(EIIa-cre)C5379Lmgd/J (EIIa-Cre mice) and B6129-Tg(Wap-cre)11738Mam/J (WAP-Cre) transgenic mice (Jackson Laboratories).

Genotype analysis. Tail tissue from 3-week-old pups was used. Where necessary, adult mice were euthanized and various tissue samples were collected. Tissue samples were digested overnight at 60°C in lysis buffer (50 mM TrisHCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, 30 mg/ml

proteinase K). DNA-containing supernatant was diluted 1:50 in 10 mM TrisCl (pH 8.0) and 2 µl of diluted supernantant was used for PCR in a PTC-2000 Peltier Thermal Cycler (MJ Research, MA, USA) a final volume of 20 µl. To assess *Bin1* genotype, amplification conditions were 94°C for a 4-min initial strand separation followed by 35 cycles at 94°C for 20s, 58°C for 1min, 72°C for 1 min, a 10-min final elongation step at 72 °C. Two oligonucleotides 5'TGGAGTCTGCCACCTTCTATCC 3' was complementary to a sequence upstream from the first loxP and 5'GCTCATACACCTCCTGAAGACAC3' downstream from the second loxP site (Integrated DNA Technologies, Inc.). The expected size were 0.9 kb, 1.07 kb and 0.31 kb for wild-type, flox, and deleted flox (floxΔ) alleles, respectively. Amplification products were separated by electroporation on 2% agarose gels prestained with ethidium bromide, using HaeIII digested φX174 phage DNA (Fisher) as a molecular size marker.

Histological analysis. Hearts from neonates that expired at birth were surgically dissected and fixed in formalin. Tissue sections were produced by standard methods and stained with hematoxylin and eosin.

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Tables

Table I. Increased mosaicism produced by use of the variant loxP site. The frequencies of recombination produced by EIIa-cre on floxed genes using wt loxP sites is taken from Lakso et al.(6).

	Frequency	
Recombination	Wild-type loxP site	Variant 3' loxP site
Complete (systemic)	50%	10%
Mosaic	50%	60%
Non-recombinant	0%	30%

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Figure Legends

Figure 1. Gene targeting strategy using a variant loxP site. White boxes represent exons and the colored arrows indicate the wild-type (solid) and variant mutant (hatched) loxP sites. Thin arrows note the location of PCR primers with the size of the predicted amplication products given in basepairs. The structure of the 'tri-lox' targeting plasmid is noted along with the structure of the desired floxed or floxΔ alleles generated by Cre-mediated recombination in ES cells *in vitro* or *in vivo*, respectively. The position of the T->C mutation introduced into the variant loxP is shown.

Figure 2. Competence of the variant loxP site to mediate tissue-specific recombination. (A.) Mammary gland-specific recombination in *Bin1* wt/flox mice that harbor a Wap-Cre transgene. Genomic DNAs isolated from various tissues were subjected to PCR analysis using the *Bin1* oligonucleotide probes depicted in Figure 1 (blue arrows). Conversion of the flox allele to the floxΔ allele occurred specifically only in mammary glands of female mice. (B.) Kinetics of Wap-Cre-mediated recombination. Generation of the floxΔ allele is apparent during and after pregnancy and weaning.

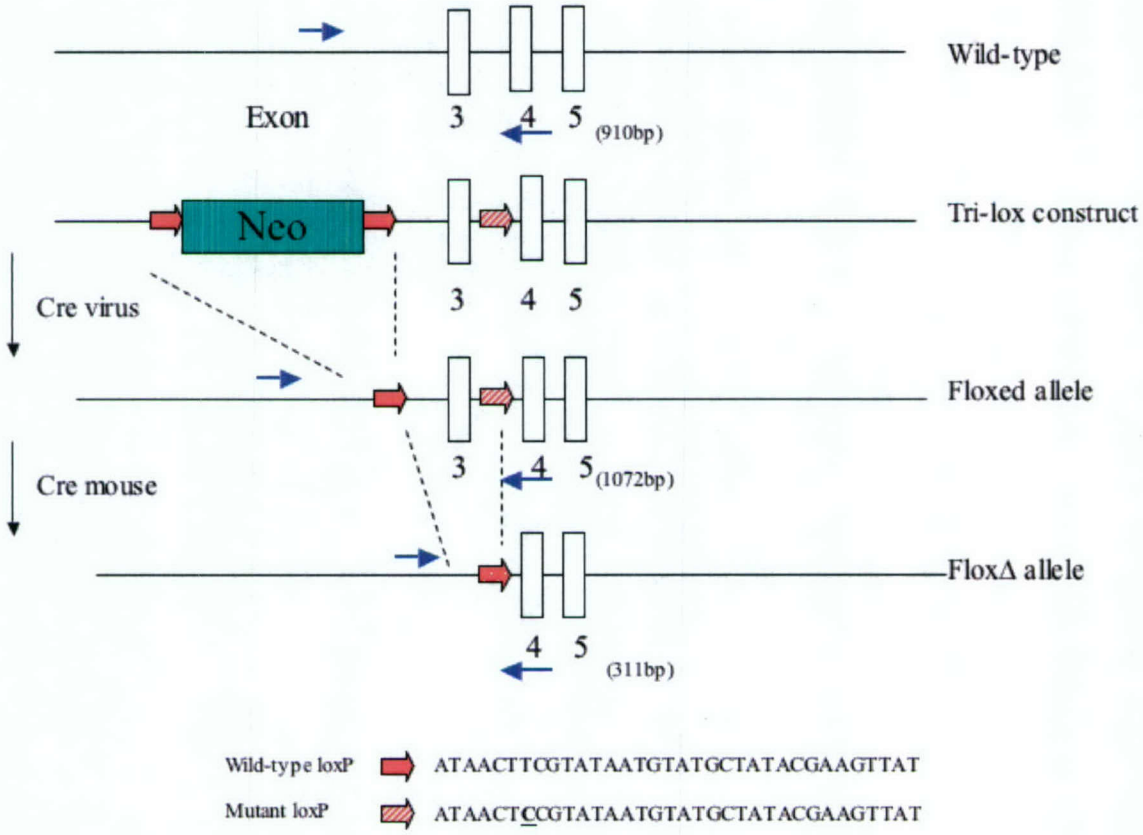
Figure 3. The floxΔ allele of *Bin1* is a functional knockout. Histological analysis of the heart from Bin1(KO)/(floxΔ) pups that expired at birth revealed severe myocardial hypertrophy indistinguishable from that seen *Bin1* null mice(10).

Figure 4. Competency of the variant loxP site to produce complete or mosaic gene knockout. Genomic DNA from mouse tails was evaluated for recombination of the floxed allele mediated by

Cre recombinase expressed from the adenoviral EIIa promoter, which is expressed from E1-E4 of development including in the 1-cell zygote(3). As depicted in Figure 1, the wild-type (wt), flox, and floxΔ allele yield PCR products of 0.90, 1.07, and 0.31 kb, respectively. The molecular weight marker is HaeIII digested ϕX174 phage DNA. Mosaic indicates the presence of both recombined and intact flox alleles.

Figure 5. Efficiency of Cre-mediated recombination is consistent across different tissues in individual mosaic mice. Two mosaic mice exhibiting a high or low degree of conversion of the flox allele to the floxΔ allele are shown (A and B). In six tissues examined, the proportion of cells with a recombined allele is consistent with prior analysis of tail biopsies.

Figure 1.


Wild-type
Exon
3
4
5
(910bp)
Neo
Tri-lox construct
Cre virus
Floxed allele
Cre mouse
(1072bp)
FloxΔ allele
(311bp)
Wild-type loxP
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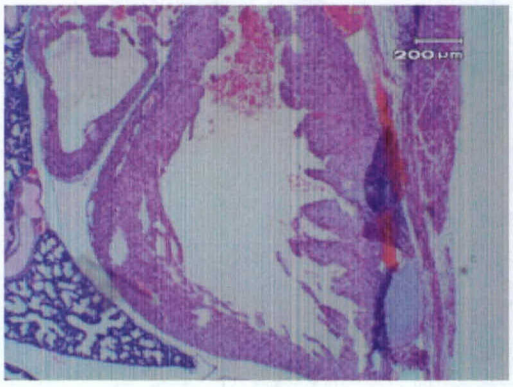
Mutant loxP
ATAACTCCGTATAATGTATGCTATACGAAGTTAT

Figure 2.

Bin1 +/+ 1 dpp



Bin1 (KO/floxΔ) 1 dpp

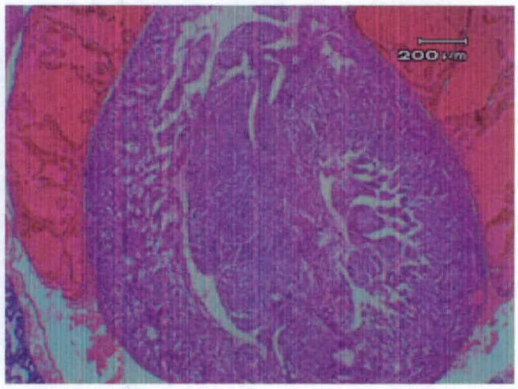


Figure 3.

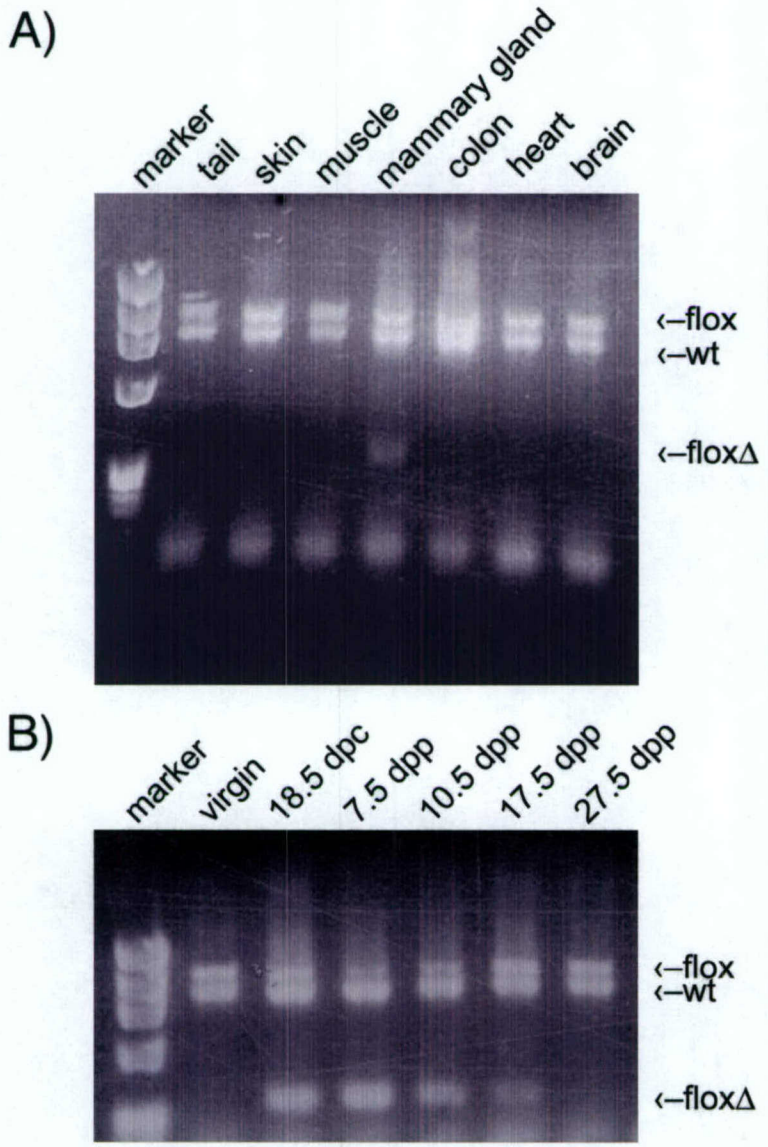

A)
marker
tail
skin
muscle
mammary gland
colon
heart
brain
‹–flox
‹–wt
‹–floxΔ
B)
marker
virgin
18.5 dpc
7.5 dpp
10.5 dpp
17.5 dpp
27.5 dpp
‹–flox
‹–wt
‹–floxΔ

Figure 4.

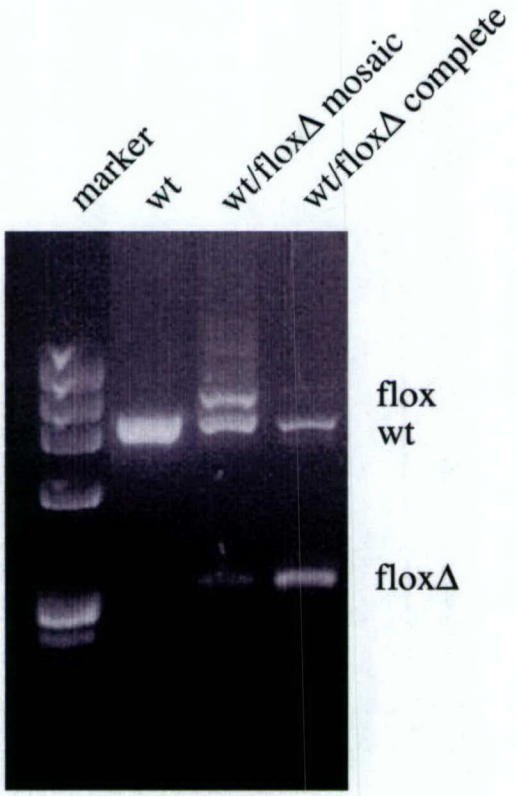
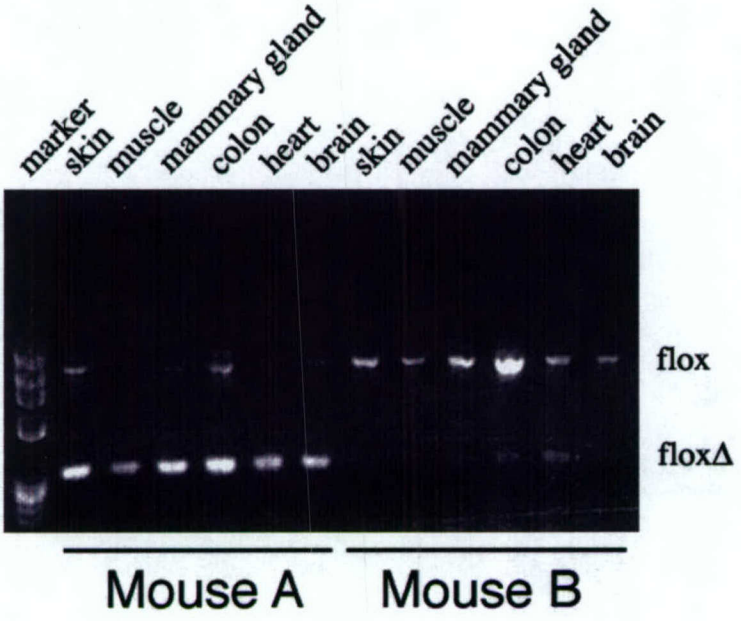


Figure 5.


marker
skin
muscle
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brain
flox
floxΔ
Mouse A
Mouse B